Identity, virulence genes, and clonal relatedness of isolates from patients with diarrhea and drinking water

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Abstract: Among 800 stool specimens from patients with diarrhea submitted by Primary Care Centers for routine analysis to the Hospital of León (NW Spain) Microbiology and Parasitology Service, 32 (4%) were tested positive for *Aeromonas* spp. Mixed infections with other enteric pathogens occurred in 12 patients. *A. caviae* was isolated from 23 clinical specimens. There also were patients infected with *A. media*, *A. hydrophila*, *A. bestiarum* and *A. veronii* biovar *veronii*. All but three isolates carried one or more of the virulence genes. The incidence of the *alt*, *hlyA*, *aerA*, *ast* and *laf* genes was 71.9, 28.1, 25.0, 18.8, and 9.4%, respectively. The *alt*+/+ combination was detected in four isolates and the *aerA*+/+/*hlyA*+/+ combination in the two *A. hydrophila* isolates. None of the strains harbored the TTSS, *stx1* or *stx2* genes and nine bore plasmids. Thirty clinical isolates and a collection of 12 *A. caviae* and *A. media* strains obtained from León municipal drinking water over the study period were typed by PFGE. PFGE patterns revealed genetic relatedness and persistence over time among water isolates and some clinical isolates. Interestingly, one *A. caviae* (*aerA*-/*hlyA*-/*alt*+/+/*ast*-/*laf*+) human isolate and two *A. caviae* (*aerA*-/*hlyA*-/*alt*+/+/*ast*-/*laf*+) drinking water isolates had indistinguishable PFGE patterns, suggesting waterborne infection.

Response to Reviewers: Answers to reviewer's comments:

* Information on the detection of TTSS gene has been included and has been taken into consideration in the "Discussion" section.

* Mistakes have been corrected and some of the suggestions of the reviewer have been included in the manuscript".

IDENTITY, VIRULENCE GENES AND CLONAL RELATEDNESS OF
Aeromonas ISOLATES FROM PATIENTS WITH DIARRHEA AND DRINKING
WATER

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Abstract

Among 800 stool specimens from patients with diarrhea submitted by Primary Care Centers for routine analysis to the Hospital of León (NW Spain) Microbiology and Parasitology Service, 32 (4%) were tested positive for *Aeromonas* spp. Mixed infections with other enteric pathogens occurred in 12 patients. *A. caviae* was isolated from patients clinical specimens. There also were patients infected with *A. media*, *A. hydrophila*, *A. bestiarum* and *A. veronii* biovar *veronii*. All but three isolates carried one or more of the virulence genes ~~tested~~. The incidence of the *alt*, *hlyA*, *aerA*, *ast* and *laf* genes was 71.9, 28.1, 25.0, 18.8, and 9.4%, respectively. The *alt*⁺/*ast*⁺ combination was detected in four isolates and the *aerA*⁺/*hlyA*⁺ combination in the two *A. hydrophila* isolates. None of the strains harbored the ~~*E. coli* genes TTSS, stx1 and stx2 genes~~ and nine bore plasmids. Thirty clinical isolates and a collection of 12 *A. caviae* and *A. media* strains obtained from León municipal drinking water over the study period were ~~sub~~typed by PFGE. PFGE patterns revealed genetic relatedness and persistence over time among water isolates and some clinical isolates. Interestingly, one *A. caviae* (*aerA*⁻/*hlyA*⁻/*alt*⁺/*ast*⁻/*laf*⁺) human isolate and two *A. caviae* (*aerA*⁻/*hlyA*⁻/*alt*⁺/*ast*⁻/*laf*⁺) drinking water isolates had indistinguishable PFGE patterns, suggesting waterborne infection.

Keywords: *Aeromonas*, stool specimens, drinking water, PFGE

Introduction

Aeromonas spp. are primarily aquatic organisms that may be readily isolated from aquatic environments, drinking water and a wide range of foods. Some species cause disease in fish, amphibians and reptiles. In humans, mesophilic *Aeromonas* are recognized as causative agents of extraintestinal diseases such as septicemia, particularly in immunocompromised hosts, wound infections and a number of less well described diseases [26]. Over the past 25 years, mesophilic *Aeromonas* have received increasing attention as an emergent agent of food borne illness. In Spain, data from the National Epidemiology Centre [16] indicate that *Aeromonas* spp. occupied the fourth place among causes of total gastrointestinal diseases reported each year over the period 1997 to 2006. Because *Aeromonas* spp. can occur in municipal drinking water supplies despite water treatment strategies, the possibility of waterborne *Aeromonas* infections in humans has been discussed [27, 55]. For this reason, some countries such as the Netherlands have set standards for *Aeromonas* spp. in drinking water [4].

Pathogenesis of *Aeromonas* spp. involves a wide group of virulence determinants, their roles not being conclusively established for any human infections. As for other bacteria, it appears that only certain intraspecific subsets of strains act as human pathogens [27, 51]. The literature shows that the occurrence of genes encoding hemolytic, cytotoxic, cytotoxic and enterotoxin activities (*aerA*, *hlyA*, *alt*, and *ast*) may contribute to diarrheal-related virulence [24, 41, 54] and that expression of peritrichous flagella encoded by the *laf* gene cluster enhance eukaryotic cells adherence and invasiveness, and also the ability to form biofilms *in vitro* [20]. Moreover, the presence of a type III secretion system (TTSS) can play a role in the virulence of motile *Aeromonas* by delivering a range of proteins (including toxins) into the host cell [56]. In

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addition, evidence of plasmid encoded expression by *Aeromonas* isolates of cytotoxins similar to Shiga-like toxins of verotoxigenic *E. coli* has been observed [23, 44].

This study was designed with two aims. The first was to determine the prevalence and ~~the identification of identity of~~ *Aeromonas* ~~species~~ in cases of gastroenteritis in a Spanish city (León, NW of the country) and to investigate the presence of virulence-associated genes in isolates from patients with diarrhea. The second aim was to establish the genetic relationships of strains from stool specimens and from León drinking water by using pulsed-field gel electrophoresis (PFGE).

Materials And Methods

Fecal specimens. From October 2005 to January 2007, 800 stool specimens submitted by Primary Care Centers to the Hospital of León Microbiology and Parasitology Service (coverage population, 351.086 inhabitants) were routinely tested for the detection of enteric pathogens (bacteria, enteric viruses and parasites) and also plated on freshly prepared m-*Aeromonas* selective agar (Merck, KGaA, Darmstadt, Germany). After incubation at 28 °C for 22 h, a total of 160 suspect colonies were screened for standard phenotypic traits: Gram stain, motility, oxidase and catalase production, fermentation of glucose and resistance to vibriostatic agent O/129 [29].

Drinking water isolates. For comparative typing by using pulse field gel electrophoresis (PFGE), we included 12 *A. caviae* and *A. media* strains (Table 3) which were isolated during the study period from León municipal drinking water meeting quality standards for indicator organisms and chlorine levels [5].

Phenotypic characterization of *Aeromonas* isolates from patients with diarrhea. A total of 32 *Aeromonas* isolates (one from each positive sample) was tested for indole production, gas from glucose, Voges Proskauer, L-arginine hydrolysis, lysine

and ornithine decarboxylase, esculin hydrolysis, susceptibility to 30 µg of cephalotin, methyl red test, acid production from m-inositol, L-arabinose, lactose, D-mannitol, salicin, D-sucrose, D-rhamnose, and D-sorbitol, gelatinase and elastase activities, and β-hemolysis on sheep blood agar plates as described elsewhere [21]. The phenotypic scheme of Abbott et al. [1] was employed for identification of isolates to *Aeromonas* Groups level. Additional tests useful in differentiating species within the *A. hydrophila* complex (*A. hydrophila*, *A. bestiarum* and *A. salmonicida*), the *A. caviae* complex (*A. caviae*, *A. media* and *A. eucrenophila*), and the Group 1 “ODC (ornithine decarboxylase) + only” (*A. veronii* biovar *veronii* and *A. allosaccharophila*) were also performed [1]. The suicide phenomenon at 30 and 37 °C was investigated as described by Namdari and Cabelli [33].

Detection of associated virulence genes. The target genes investigated in this study, the sequence of each primer, the annealing temperatures, the expected PCR products and their references are listed in Table 1.

Genomic DNA was extracted from exponential cultures of the organisms in Trypticase soy broth (Becton Dickinson S.A., Madrid, Spain) taking into account ISO 22174 general requirements and according to ISO 20837 [6, 7]. PCR amplification and detection of PCR products were performed according to the general requirements described in ISO 22174 and ISO 20838 [6, 8]. PCR conditions for the *aerA*, *hlyA*, *alt*, *ast*, *laf*, *ascF-G*, *stx1* and *stx2* genes have been described elsewhere [12, 30, 34, 37]. Aliquots from each reaction were analyzed by 2% agarose gel electrophoresis and viewed after ethidium bromide staining under UV light.

Plasmids detection. Plasmid DNA isolation was performed with the Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories, Hercules, CA) according to the

manufacturer's instructions and analyzed by agarose gel electrophoresis (1.5%) at 100 V for 40 min.

PFGE. The standardized PulseNet PFGE protocol for pathogenic Gram-negative enteric bacteria [36] and a CHEF-DR III apparatus (Bio-Rad) were used for PFGE analysis. DNA plugs were digested with *Xba*I restriction enzyme (New England Biolabs, Ipswich, MA, USA). Strains were typed at least at two separate occasions. After electrophoresis, the gels were stained with ethidium bromide, and documented with the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Kodak Co., Rochester, NY). The banding patterns were analyzed with the GelCompar II program (version 5.1., Applied Maths, St.-Martens-Latem, Belgium). Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA). Isolates were considered genetically “indistinguishable” if their PFGE patterns were 100% similar and “closely related” if they showed more than 85% similarity, corresponding to one to three band differences [48]. Isolates with PFGE profiles showing less than 85% similarity were defined as being genetically unrelated strains. A similarity threshold of >85% defined clusters.

Results

Of the 800 stool samples evaluated, 32 samples from different patients were positive for *Aeromonas* spp. with an overall positivity rate of 4%. There were 17 positive samples when the mean monthly environmental temperature was below 12 °C and 15 when the mean monthly environmental temperature ranged between 12 and 24.3 °C (<http://clima.meteored.com/clima-en-Europa.html>).

Information about patients and *Aeromonas* isolates recovered from their stools is shown in Table 2. All positive samples except one were from outpatients. Of the 32

selected *Aeromonas* isolates (one from each patient), 26 were allocated to the *A. caviae* complex, four to the *A. hydrophila* complex and two to the “ODC+ only” Group 1. The species found were *A. caviae* (23 patients, 71.9%), *A. media* (3 patients, 9.4%), *A. hydrophila* (2 patients, 6.2%), *A. bestiarum* (2 patients, 6.2%), and *A. veronii* biovar *veronii* (2 patients, 6.2%). Mixed infections occurred in 12 patients (37.5%), and other pathogens that were found concurrently with *A. caviae* or *A. media* included *Campylobacter* (6 patients, 18.7%), *Salmonella* (4 patients, 12.5%) and *Giardia lamblia* (1 patient, 3.1%). *Ascaris lumbricoides* was present with *A. media* and *Campylobacter* in one patient (3.1%). One further patient was a 3 years old boy with GHD (growth hormone deficiency).

Information from questionnaires completed by physicians reported that 27 patients had acute diarrhea without vomiting, two had chronic diarrhea, and three had diarrhea, vomits and fever. Stool samples from pediatric patients (up to 14 years old) and males accounted for 68.7% and 56.2% of the *Aeromonas* isolates, respectively. The 27 patients with acute diarrhea were infected with strains of the five *Aeromonas* species found, 12 of them being simultaneously positive for other enteric pathogens (Table 2). Patients with chronic diarrhea and those with diarrhea, vomits and fever were infected with *A. caviae* alone and one with *A. hydrophila* alone.

The distribution of associated virulence genes among the *Aeromonas* diarrheal isolates is summarized in Table 3. None of the strains harbored the genes *ascF-G*, *stx1* or *stx2* and nine bore plasmids. β -hemolysis on sheep blood agar plates was only displayed by isolates carrying the *aerA* and/or the *hlyA* genes, the remaining isolates showing zones of incomplete hemolysis.

Two human isolates (H8 and H9) were consistently non-typeable with the PFGE applied method neither by using a second enzyme (*SpeI*). PFGE following *XbaI*

digestion identified 30 patterns whose profiles differed by at least one band. The dendrogram obtained (Fig. 1) showed a cluster (cluster B) of eight *A. media* water strains with 100% similarity. The remaining water isolates (*A. caviae*) grouped together in cluster G, two of them (W73 and W74) being indistinguishable from strain H13 recovered from a 51 years old female, without apparent underlying medical complications, who was negative for other enteric pathogens. There were two clusters with two human isolates with identical *Xba*I PFGE profiles: two identified as *A. bestiarum* (cluster A) and two as *A. caviae* (cluster F). Other clusters (C, D, E and H) included nine closely related *A. caviae* isolates from patients with diarrhea. Indistinguishable isolates possessed identical virulence genes.

Discussion

One of the most commonly reported human infections by *Aeromonas* spp. is gastroenteritis. Clinical manifestations of disease can range from mild self-limiting watery diarrhea to a more severe and invasive dysenteric form. Chronic diarrheal episodes and isolated cases of a cholera-like illness have also been described [26]. In several reported studies throughout the world, *Aeromonas* spp. have been isolated at a rate of 0.6 to 7.2% from patients with diarrhea predominantly from infants and children [3, 10, 18, 31, 43, 53]. The highest rate, 52.4%, was detected in Peruvian infants with severe diarrhea although 57.6% of the *Aeromonas*-positive patients had mixed infections with other enteric pathogens [35]. A similar high frequency of co infection has been found in Bangladesh and India [3, 43]. Our results are in agreement with those found in most of the above surveys. Thus, *Aeromonas* spp. was isolated from 4.0% of the patients, most of them being infants (46.9%) and other pediatric patients (21.9%). Clinically, acute illness was the most common symptom whereas fever, vomiting or

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4 181 chronic diarrhea were present in only a few cases. In addition, common enteric
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6 182 pathogens other than *Aeromonas* [spp.](#) were recovered from more than one third of
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8 183 diarrheic stools. Because of the ubiquitous nature of *Aeromonas* spp. in raw foods and
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10 184 water, it has been argued that in mixed infections these bacteria are transient colonizer
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12 185 without causal relationship with disease, but also that multiple pathogens may act
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14 186 synergistically to produce diarrhea [3, 35]. Unlike most surveys that have found peaks
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16 187 of *Aeromonas*-associated gastroenteritis in warmer months, we did not observe seasonal
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18 188 patterns for isolation.

19 189 The majority of our isolates (71.9%), which were identified to phenospecies with
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21 190 biochemical schemes [1] and later confirmed by molecular methods (AFLP and *gyrB*
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23 191 gene sequences; data not shown), belonged to the species *A. caviae*. There also were
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25 192 some isolates of *A. media*, *A. hydrophila*, *A. bestiarum* and *A. veronii* biovar *veronii*. In
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27 193 several studies conducted in Europe, the United States and India, *A. caviae* was
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29 194 dominant followed by *A. hydrophila* and *A. veronii* biovar *sobria* [3, 10, 18, 31, 43, 53].
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31 195 This agrees with an earlier report from Spain [49], which noted that, between 1989 and
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33 196 1999, the three species accounted for 90% of all *Aeromonas*-associated gastroenteritis.
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35 197 Other studies in Brazil, Thailand and India [28, 51] reported *A. hydrophila* as
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37 198 predominant, and a high isolation rate of *A. trota* has also been found [3]. These
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39 199 differences may depend on the geographical location but also on other factors at least in
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41 200 part related to the isolation and identification methods used. Thus, as most *Aeromonas*
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43 201 culture media contain ampicillin, the high incidence of *A. trota*, an ampicillin-
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45 202 susceptible species, found by the latter authors was attributed to the isolation technique
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47 203 used.

48 204 *A. media* is isolated from the environment and occasionally from patients with
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50 205 diarrhea [3], but its clinical significance is not known [29] although Singh [42] reported

that regardless of their source, *A. media* strains are potentially enterotoxigenic. In this study, two of the *A. media* positive patients had concurrent infection with *Salmonella*. *A. veronii* biovar *veronii* and *A. bestiarum* have rarely been isolated from humans and little information is available regarding its occurrence in clinical specimens; however, two strains of each species were isolated by us as the sole pathogen from two children and two elderly men, and also from gastroenteritis in Switzerland, India and Brazil [14, 25, 43].

The pathogenicity of *Aeromonas* spp. is multifactorial and complex and may involve the products of a number of different genes acting either alone or in concert [19, 40]. One rational approach to determine whether *Aeromonas* strains have the potential to be virulent is to screening for an array of virulence-related genes [2, 52]. A potent virulence factor of *Aeromonas* [strains](#) is the cytotoxic enterotoxin Act, which is closely related to aerolysin (AerA), both of them being hemolytic, cytotoxic, enterotoxic and lethal for mice. *Aeromonas* may also produce heat labile (Alt) and heat stable (Ast) cytotoxic enterotoxins [19]. Sha et al. [41] established a role for the three enterotoxins in causing gastroenteritis in a mouse model by developing isogenic mutants with the major contribution from Act, followed by the Alt and Ast. Results from Albert et al. [3] suggest that the product of both the *alt* and *ast* genes may act synergistically to induce severe diarrhea.

The incidence of the *aerA*, *alt* and *ast* genes in our clinical isolates was 25, 71.9 and 18.8%, respectively, the *alt*⁺/*ast*⁺ combination being detected in four of them (12.5%). These findings are comparable with those of Sinha et al. [43] who, in isolates from Indian patients with acute diarrhea, found that *act/aerA* accounted for 30%, *alt* was dominant and *ast* was the less prevalent. Overall, among fecal isolates from Mexico and Catalonia (Spain), Aguilera-Arreola et al. [2] reported that genes for the aerolysin-

hemolysin, Alt, Ast and Alt + Ast toxins accounted for 89.0%, 34.9%, 28.4% and 7.3%, respectively. Among their *A. caviae* isolates, 90.2% carried the genes encoding cytolytic activity whereas the *alt* gene was present in only 9.8%. By contrast, we have detected in *A. caviae* an occurrence of the *aerA* and *alt* genes of 21.7 and 73.9%, respectively. A survey in Bangladesh [3] found absence of the *act* gene alone and a high incidence of the *alt* and *alt*⁺/*ast*⁺ genes in isolates from children with diarrhea.

In addition to the enterotoxigenic *Act/aerA*, which causes β -hemolysis, a β -hemolytic *Vibrio cholerae*-like hemolysin (HlyA) has been described. The *aerA*⁺/*hlyA*⁺ genotype and in some cases the *aerA*⁺/*hlyA*⁻ genotype have been reported to be reasonable predictors of human diarrheal disease [24]. Combination of both genes, which are less common in *A. caviae* than in *A. hydrophila*, was only present in the two *A. hydrophila* isolates. In an earlier study [22], we found that strains belonging to the genotypes *aerA*⁺/*hlyA*⁺, *aerA*⁺/*hlyA*⁻ and *aerA*⁻/*hlyA*⁻ were enterotoxigenic in the suckling mouse test and had the capability to express other virulence traits (proteolytic, hemolytic and cytotoxic activities) at different temperatures.

Swarming motility, a lateral flagella dependent behavior, has been implicated in biofilms formation and adhesion to eukaryotic cells. The gene cluster *laf* responsible for *Aeromonas* lateral flagella production [20] was detected in three human isolates (9.4%) which is lower than the incidence (36.7%) found by Aguilera-Arriola et al. [2]. However, all of our drinking water isolates carried the *laf* gene, two of them (W73 and W74) being, by PFGE, indistinguishable from the human isolate H13 (Fig 1).

In this study, there were three *A. caviae* strains that did not amplify any of the genes tested although they showed incomplete hemolysis and two were recovered as the only suspect pathogen. Negative PCR results do not exclude pathogenicity because at least some *Aeromonas* strains possess a wide range of virulence factors, other than those

tested by us, that enable them to overcome host defenses and establish infection. Among these factors are surface molecules, different toxins, many enzymes, iron acquisition systems, protein secretion systems and novel virulence determinants that may play an important role in pathogenesis [19, 39, 40].

Our results and those from others suggest that in *Aeromonas* clinical isolates the distribution pattern of associated-virulence genes varies depending on the species and geographical location and also that despite current knowledge of *Aeromonas* virulence determinants, there is still no way to identify a given clinical or environmental isolate that has the capacity to cause human illness although some data indicate a role of both the bacterial virulence factors and host-responses [19].

The presence of TTSS seems to be related to the pathogenicity of some *Aeromonas* strains [50, 56]. However, it is more frequently found in *A. hydrophila* and *A. veronii* strains than in *A. caviae* isolates, even from clinical specimens [50]. This fact can explain the lack of detection of TTSS genes in our isolates, as they belong mainly to the species *A. caviae*.

Human infections with *Aeromonas* spp. have occasionally been associated with hemolytic uremic syndrome (HUS) [17], a life-threatening condition normally associated with enterohemorrhagic *E. coli* (EHEC) strains producing the cytotoxins Stx1 and/or Stx2. The *stx1* gene has been detected in clinical and environmental isolates of *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* [23, 44]. As this could explain reports on HUS related to *Aeromonas* spp., we investigated the presence of *stx1* and *stx2* genes and plasmids. None of our isolates were PCR positive for the *stx* genes and 28% harbored plasmids. Although most strains of *A. salmonicida* carry at least one large plasmid, plasmids are detected less commonly (15 to 30%) in human diarrhea-associated *Aeromonas* isolates [11].

PFGE, which has remarkable discriminatory power and reproducibility, has been successfully applied to the subtyping of many pathogenic bacteria [9]. Over the last years, a number of studies using PFGE and other molecular methods have been used for comparative typing of clinical and/or environmental *Aeromonas* strains [10, 15, 32, 45-47] most of them concluding: (i) that specific clones colonized certain aquatic ecosystems such as drinking water supplies probably as consequence of biofilms development, (ii) that there was not similarity among clinical isolates, and (iii) that stools and drinking water isolates were genetically unrelated. In one of the earliest studies, following a number of cases of diarrhea in children using a small community water supply, Moyer et al. [32] reported colonization of the water supply with *Aeromonas* identical ribotypes, but unique ribotypes in *Aeromonas* patient strains, concluding that water was not the source of *Aeromonas* infections. Similar results were obtained in French studies using RAPD and PFGE for typing isolates from hospital-acquired infections and from the hospital water supply [46, 47]. De la Morena et al. [15] studied *Aeromonas* isolates recovered from children involved in two outbreaks of gastroenteritis in day-care centers, finding that the PFGE patterns differed in all except two strains. Also by PFGE, *Aeromonas* isolates from patients with diarrhea and groundwater isolates from private wells throughout Wisconsin were genetically unrelated [10]. By using RAPD, REP-PCR and ERIC-PCR, Szczuka and Kaznowski [45] found that strains isolated from patients in Poland, different parts of Europe and Asia were different clones and that there was not genetic similarity between clinical and environmental strains although the persistence of a single clone in a city water distribution was observed.

In this study, PFGE analysis of the water isolates revealed persistence of two indistinguishable or closely related (up to three band differences) clones of *A. media* and

A. caviae in the water distribution system of León over the 16 months study period. For clinical isolates, different PFGE patterns were present in stool specimens, some of them grouping in clusters of indistinguishable or closely related strains. Interestingly, there was no clear relation among the origin of isolates or time of isolation; for instance, the two *A. caviae* isolates in cluster C were recovered in October 2005 and May 2006, those in cluster D in February and October 2006 and those in cluster E in April 2006 and January 2007. The indistinguishable isolates grouped in clusters A and F were detected in the same months (March and June 2006) but from samples obtained in four different Primary Care Centers. It is also noteworthy that one *A. caviae* clinical isolate and two *A. caviae* water isolates with identical pattern of associated-virulence genes (*aerA*⁻/*hlyA*⁻/*alt*⁺/*ast*⁺/*laf*⁺) showed identical pulsotype providing a link between *Aeromonas* spp. and diarrheal disease. Chopra [13] also reported that two pairs of *A. caviae*/*A. media* isolates from water and patients carrying only one of the three enterotoxin genes (*alt*) had indistinguishable PFGE patterns, the author indicating that biochemical identification of isolates, determination of virulence traits and DNA fingerprinting could provide necessary information on the pathogenic potential of *Aeromonas* spp. that can cause waterborne infections.

In summary, infection with *Aeromonas* spp., mainly *A. caviae*, occurred throughout the year and was significantly more common in pediatric patients. Acute diarrhea was the predominant clinical feature, with the majority of isolates harboring associated-virulence genes. PFGE of human and water isolates, which were recovered from the same geographic area during the same time period, revealed genetic relatedness and persistence over time in water and some clinical isolates. Interestingly, one human isolate had indistinguishable PFGE pattern and identical virulence pattern than two water isolates, suggesting waterborne infection.

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Table 1 PCR primers, annealing temperatures and expected PCR products for virulence associated genes

Target Genes	Primers' sequences (5'-3')	Annealing temperature (°C)	Produced amplicon length (bp)	References
<i>aerA</i>	GC(A/T)GA(A/G)CCC(A/G)TCTATCC(A/T)G TTTCTCCGGTAACAGGATTG	55	252	Santos et al. [38]
<i>hlyA</i>	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	62	597	Wong et al. [54]
<i>alt</i>	CCA TCC CCA GCC TTT ACG CCA T TTT CAC CGA GGT GAC GCC GT	63	338	Martínez et al. [30]
<i>ast</i>	ATG CAC GCA CGT ACC GCC AT ATC CGG TCG TCG CTC TTG GT	66	260	Martínez et al. [30]
<i>laf</i>	GGT CTG CGC ATC CAA CTC GCT CCA GAC GGT TGA TG	60	550	Gavín et al. [20]
<i>ascF-G</i>	ATG AGG TCA TCT GCT CGC GC GGA GCA CAA CCA TGG CTG AT	55	ca 1 Kb	Chacón, 2004
<i>stx₁</i>	ATA AAT TGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC	61	180	Paton & Paton [34]
<i>stx₂</i>	GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G	61	255	Paton & Paton [34]

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Table 2 Symptoms, age and gender of patients suffering from gastroenteritis associated with *Aeromonas* spp.

Symptoms	Age (years)					Species	No. of patients	Other organisms	No. of patients
	Total patients	≤2	>2-14	>14-65	>65				
Acute diarrhea	27	12	6	4	5				
Gender (F/M) ^a	(10/17)	(5/7)	(2/4)	(2/2)	(1/4)				
						<i>A. caviae</i>	19		
						<i>A. media</i>	3		
						<i>A. bestiarum</i>	2		
						<i>A. veronii</i> biovar <i>veronii</i>	2		
						<i>A. hydrophila</i>	1		
								<i>Campylobacter</i>	6
								<i>Salmonella</i>	4
								<i>Campylobacter</i> + <i>Ascaris</i>	1
								<i>Giardia lamblia</i>	1
Chronic diarrhea	2	2							
Gender (F/M)	(2/0)	(2/0)							
						<i>A. caviae</i>	2		
Vomits/diarrhea/fever	3	1	1		1				
Gender (F/M) ^a	(2/1)	(0/1)	(1/0)		(1/0)				
						<i>A. caviae</i>	2		
						<i>A. hydrophila</i>	1		

^aF, female; M, male

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Table 3 ~~Identity and d~~Distribution of associated virulence genes among 32 *Aeromonas* isolates from human stool specimens and 12 *Aeromonas* isolates from drinking water

Species	Total isolates	<i>aerA</i>	<i>hlyA</i>	<i>alt</i>	<i>ast</i>	<i>laf</i>	No of isolates
<u>Stool specimens</u>							
<i>A. caviae</i>	23	-	-	+	-	-	8
		+	-	+	-	-	4
		-	-	+	-	+	2
		-	+	+	-	-	2
		-	+	+	+	-	1
		+	-	-	-	-	1
		-	+	-	-	-	1
		-	-	-	+	-	1
		-	-	-	-	-	3
<i>A. media</i>	3	-	-	+	-	-	2
		-	+	+	-	-	1
<i>A. hydrophila</i>	2	+	+	+	+	-	1
		+	+	-	+	-	1
<i>A. bestiarum</i>	2	-	+	+	+	-	2
<i>A. veronii</i> biovar <i>veronii</i>	2	+	-	-	-	-	1
		-	-	-	-	+	1
<u>Drinking water</u>							
<i>A. media</i>	8	-	-	+	-	+	8
<i>A. caviae</i>	4	+	-	+	-	+	2
		-	-	+	-	+	2

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Fig. 1 Genetic similarity based on PFGE of *Aeromonas* strains from patients with diarrhea and drinking water. The horizontal upper bar represents genetic similarity (%). Letters together strain number represent: W, isolates from water samples and H, isolates from clinical stools