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

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### ► To cite this version:

M. Pablos, M.-A. Remacha, J.-M. Rodríguez-Calleja, J. A. Santos, A. Otero, et al.. Identity, virulence genes, and clonal relatedness of isolates from patients with diarrhea and drinking water. *European Journal of Clinical Microbiology and Infectious Diseases*, 2010, 29 (9), pp.1163-1172. 10.1007/s10096-010-0982-3 . hal-00600040

**HAL Id: hal-00600040**

**<https://hal.science/hal-00600040>**

Submitted on 13 Jun 2011

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Editorial Manager(tm) for European Journal of Clinical Microbiology & Infectious  
Diseases  
Manuscript Draft

Manuscript Number: EJCMIID-D-09-00648R1

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

Article Type: Article

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

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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*+/*ast*+ 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".

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4 1 IDENTITY, VIRULENCE GENES AND CLONAL RELATEDNESS OF  
5 2 *Aeromonas* ISOLATES FROM PATIENTS WITH DIARRHEA AND DRINKING  
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4 17 **Abstract**

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6 18       Among 800 stool specimens from patients with diarrhea submitted by Primary  
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8 19 Care Centers for routine analysis to the Hospital of León (NW Spain) Microbiology and  
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10 20 Parasitology Service, 32 (4%) were tested positive for *Aeromonas* spp. Mixed infections  
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12 21 with other enteric pathogens occurred in 12 patients. *A. caviae* was isolated from 23  
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14 22 patientsclinical specimens. There also were patients infected with *A. media*, *A.*  
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16 23 *hydrophila*, *A. bestiarum* and *A. veronii* biovar *veronii*. All but three isolates carried one  
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18 24 or more of the virulence genes tested. The incidence of the *alt*, *hlyA*, *aerA*, *ast* and *laf*  
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20 25 genes was 71.9, 28.1, 25.0, 18.8, and 9.4%, respectively. The *alt*<sup>+</sup>/*ast*<sup>+</sup> combination was  
21  
22 26 detected in four isolates and the *aerA*<sup>+</sup>/*hlyA*<sup>+</sup> combination in the two *A. hydrophila*  
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24 27 isolates. None of the strains harbored the ~~*E. coli* genes TTSS, stx1 and/or stx2 genes~~ and  
25  
26 28 nine bore plasmids. Thirty clinical isolates and a collection of 12 *A. caviae* and *A. media*  
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28 29 strains obtained from León municipal drinking water over the study period were  
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30 30 subtyped by PFGE. PFGE patterns revealed genetic relatedness and persistence over  
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32 31 time among water isolates and some clinical isolates. Interestingly, one *A. caviae* (*aerA*<sup>-</sup>  
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34 32 */hlyA*<sup>-</sup>/*alt*<sup>+</sup>/*ast*<sup>-</sup>/*laf*<sup>+</sup>) human isolate and two *A. caviae* (*aerA*<sup>-</sup>/*hlyA*<sup>-</sup>/*alt*<sup>+</sup>/*ast*<sup>-</sup>/*laf*<sup>+</sup>) drinking  
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36 33 water isolates had indistinguishable PFGE patterns, suggesting waterborne infection.

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38  
39 34 **Keywords:** *Aeromonas*, stool specimens, drinking water, PFGE  
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35 **Introduction**

36 *Aeromonas* spp. are primarily aquatic organisms that may be readily isolated  
37 from aquatic environments, drinking water and a wide range of foods. Some species  
38 cause disease in fish, amphibians and reptiles. In humans, mesophilic *Aeromonas* are  
39 recognized as causative agents of extraintestinal diseases such as septicemia,  
40 particularly in immunocompromised hosts, wound infections and a number of less well  
41 described diseases [26]. Over the past 25 years, mesophilic *Aeromonas* have received  
42 increasing attention as an emergent agent of food borne illness. In Spain, data from the  
43 National Epidemiology Centre [16] indicate that *Aeromonas* spp. occupied the fourth  
44 place among causes of total gastrointestinal diseases reported each year over the period  
45 1997 to 2006. Because *Aeromonas* spp. can occur in municipal drinking water supplies  
46 despite water treatment strategies, the possibility of waterborne *Aeromonas* infections in  
47 humans has been discussed [27, 55]. For this reason, some countries such as the  
48 Netherlands have set standards for *Aeromonas* spp. in drinking water [4].

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

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4 59 addition, evidence of plasmid encoded expression by *Aeromonas* isolates of cytotoxins  
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6 60 similar to Shiga-like toxins of verotoxigenic *E. coli* has been observed [23, 44].

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8 61 This study was designed with two aims. The first was to determine the  
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10 62 prevalence and ~~the identification of identity of~~ *Aeromonas* species in cases of  
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12 63 gastroenteritis in a Spanish city (León, NW of the country) and to investigate the  
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14 64 presence of virulence-associated genes in isolates from patients with diarrhea. The  
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16 65 second aim was to establish the genetic relationships of strains from stool specimens  
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18 66 and from León drinking water by using pulsed-field gel electrophoresis (PFGE).

## 21 67 **Materials And Methods**

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23 68 **Fecal specimens.** From October 2005 to January 2007, 800 stool specimens  
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25 69 submitted by Primary Care Centers to the Hospital of León Microbiology and  
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27 70 Parasitology Service (coverage population, 351.086 inhabitants) were routinely tested  
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29 71 for the detection of enteric pathogens (bacteria, enteric viruses and parasites) and also  
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31 72 plated on freshly prepared m-*Aeromonas* selective agar (Merck, KGaA, Darmstadt,  
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33 73 Germany). After incubation at 28 °C for 22 h, a total of 160 suspect colonies were  
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35 74 screened for standard phenotypic traits: Gram stain, motility, oxidase and catalase  
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37 75 production, fermentation of glucose and resistance to vibriostatic agent O/129 [29].

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39 76 **Drinking water isolates.** For comparative typing by using pulse field gel  
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41 77 electrophoresis (PFGE), we included 12 *A. caviae* and *A. media* strains (Table 3) which  
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43 78 were isolated during the study period from León municipal drinking water meeting  
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45 79 quality standards for indicator organisms and chlorine levels [5].

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47 80 **Phenotypic characterization of *Aeromonas* isolates from patients with**  
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49 81 **diarrhea.** A total of 32 *Aeromonas* isolates (one from each positive sample) was tested  
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51 82 for indole production, gas from glucose, Voges Proskauer, L-arginine hydrolysis, lysine

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4 83 and ornithine decarboxylase, esculin hydrolysis, susceptibility to 30 µg of cephalotin,  
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6 84 methyl red test, acid production from m-inositol, L-arabinose, lactose, D-mannitol,  
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8 85 salicin, D-sucrose, D-rhamnose, and D-sorbitol, gelatinase and elastase activities, and β-  
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10 86 hemolysis on sheep blood agar plates as described elsewhere [21]. The phenotypic  
11  
12 87 scheme of Abbott et al. [1] was employed for identification of isolates to *Aeromonas*  
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14 88 Groups level. Additional tests useful in differentiating species within the *A. hydrophila*  
15  
16 89 complex (*A. hydrophila*, *A. bestiarum* and *A. salmonicida*), the *A. caviae* complex (*A.*  
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18 90 *caviae*, *A. media* and *A. eucrenophila*), and the Group 1 “ODC (ornithine  
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20 91 decarboxylase) + only” (*A. veronii* biovar *veronii* and *A. allosaccharophila*) were also  
21  
22 92 performed [1]. The suicide phenomenon at 30 and 37 °C was investigated as described  
23  
24 93 by Namdari and Cabelli [33].

25 94 **Detection of associated virulence genes.** The target genes investigated in this  
26  
27 95 study, the sequence of each primer, the annealing temperatures, the expected PCR  
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29 96 products and their references are listed in Table 1.

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31 97 Genomic DNA was extracted from exponential cultures of the organisms in  
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33 98 Trypticase soy broth (Becton Dickinson S.A., Madrid, Spain) taking into account ISO  
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35 99 22174 general requirements and according to ISO 20837 [6, 7]. PCR amplification and  
36  
37 100 detection of PCR products were performed according to the general requirements  
38  
39 101 described in ISO 22174 and ISO 20838 [6, 8]. PCR conditions for the *aerA*, *hlyA*, *alt*,  
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41 102 *ast*, *laf*, *ascF-G*, *stx1* and *stx2* genes have been described elsewhere [12, 30, 34, 37].  
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43 103 Aliquots from each reaction were analyzed by 2% agarose gel electrophoresis and  
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45 104 viewed after ethidium bromide staining under UV light.

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47 105 **Plasmids detection.** Plasmid DNA isolation was performed with the Quantum  
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49 106 Prep Plasmid Miniprep kit (Bio-Rad Laboratories, Hercules, CA) according to the

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4 107 manufacturer's instructions and analyzed by agarose gel electrophoresis (1.5%) at 100 V  
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6 108 for 40 min.

7 109 **PFGE.** The standardized PulseNet PFGE protocol for pathogenic Gram-negative  
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9 110 enteric bacteria [36] and a CHEF-DR III apparatus (Bio-Rad) were used for PFGE  
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11 111 analysis. DNA plugs were digested with *Xba*I restriction enzyme (New England  
12  
13 112 Biolabs, Ipswich, MA, USA). Strains were typed at least at two separate occasions.  
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15 113 After electrophoresis, the gels were stained with ethidium bromide, and documented  
16  
17 114 with the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290,  
18  
19 115 Kodak Co., Rochester, NY). The banding patterns were analyzed with the GelCompar II  
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21 116 program (version 5.1., Applied Maths, St.-Martens-Latem, Belgium). Similarity  
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23 117 between fingerprints was calculated with the Dice coefficient. Cluster analysis was  
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25 118 performed using the unweighted pair-group method with average linkages (UPGMA).  
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27 119 Isolates were considered genetically “indistinguishable” if their PFGE patterns were  
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29 120 100% similar and “closely related” if they showed more than 85% similarity,  
30  
31 121 corresponding to one to three band differences [48]. Isolates with PFGE profiles  
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33 122 showing less than 85% similarity were defined as being genetically unrelated strains. A  
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35 123 similarity threshold of >85% defined clusters.

## 36 37 38 124 **Results**

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40 125 Of the 800 stool samples evaluated, 32 samples from different patients were  
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42 126 positive for *Aeromonas* spp. with an overall positivity rate of 4%. There were 17  
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44 127 positive samples when the mean monthly environmental temperature was below 12 °C  
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46 128 and 15 when the mean monthly environmental temperature ranged between 12 and 24.3  
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48 129 °C (<http://clima.meteored.com/clima-en-Europa.html>).

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50 130 Information about patients and *Aeromonas* isolates recovered from their stools is  
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52 131 shown in Table 2. All positive samples except one were from outpatients. Of the 32  
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4 132 selected *Aeromonas* isolates (one from each patient), 26 were allocated to the *A. caviae*  
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6 133 complex, four to the *A. hydrophila* complex and two to the “ODC+ only” Group 1.  
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8 134 The species found were *A. caviae* (23 patients, 71.9%), *A. media* (3 patients, 9.4%), *A.*  
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10 135 *hydrophila* (2 patients, 6.2%), *A. bestiarum* (2 patients, 6.2%), and *A. veronii* biovar  
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12 136 *veronii* (2 patients, 6.2%). Mixed infections occurred in 12 patients (37.5%), and other  
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14 137 pathogens that were found concurrently with *A. caviae* or *A. media* included  
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16 138 *Campylobacter* (6 patients, 18.7%), *Salmonella* (4 patients, 12.5%) and *Giardia lamblia*  
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18 139 (1 patient, 3.1%). *Ascaris lumbricoides* was present with *A. media* and *Campylobacter*  
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20 140 in one patient (3.1%). One further patient was a 3 years old boy with GHD (growth  
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22 141 hormone deficiency).

23 142 Information from questionnaires completed by physicians reported that 27  
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25 143 patients had acute diarrhea without vomiting, two had chronic diarrhea, and three had  
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27 144 diarrhea, vomits and fever. Stool samples from pediatric patients (up to 14 years old)  
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29 145 and males accounted for 68.7% and 56.2% of the *Aeromonas* isolates, respectively. The  
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31 146 27 patients with acute diarrhea were infected with strains of the five *Aeromonas* species  
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33 147 found, 12 of them being simultaneously positive for other enteric pathogens (Table 2).  
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35 148 Patients with chronic diarrhea and those with diarrhea, vomits and fever were infected  
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37 149 with *A. caviae* alone and one with *A. hydrophila* alone.

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39 150 The distribution of associated virulence genes among the *Aeromonas* diarrheal  
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41 151 isolates is summarized in Table 3. None of the strains harbored the genes *ascF-G*, *stx1*  
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43 152 or *stx2* and nine bore plasmids.  $\beta$ -hemolysis on sheep blood agar plates was only  
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45 153 displayed by isolates carrying the *aerA* and/or the *hlyA* genes, the remaining isolates  
46  
47 154 showing zones of incomplete hemolysis.

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49 155 Two human isolates (H8 and H9) were consistently non-typeable with the PFGE  
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51 156 applied method neither by using a second enzyme (*SpeI*). PFGE following *XbaI*

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4 157 digestion identified 30 patterns whose profiles differed by at least one band. The  
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6 158 dendrogram obtained (Fig. 1) showed a cluster (cluster B) of eight *A. media* water  
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8 159 strains with 100% similarity. The remaining water isolates (*A. caviae*) grouped together  
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10 160 in cluster G, two of them (W73 and W74) being indistinguishable from strain H13  
11  
12 161 recovered from a 51 years old female, without apparent underlying medical  
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14 162 complications, who was negative for other enteric pathogens. There were two clusters  
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16 163 with two human isolates with identical *Xba*I PFGE profiles: two identified as *A.*  
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18 164 *bestiarum* (cluster A) and two as *A. caviae* (cluster F). Other clusters (C, D, E and H)  
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20 165 included nine closely related *A. caviae* isolates from patients with diarrhea.  
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22 166 Indistinguishable isolates possessed identical virulence genes.  
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## 25 167 **Discussion**

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27 168 One of the most commonly reported human infections by *Aeromonas* spp. is  
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29 169 gastroenteritis. Clinical manifestations of disease can range from mild self-limiting  
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31 170 watery diarrhea to a more severe and invasive dysenteric form. Chronic diarrheal  
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33 171 episodes and isolated cases of a cholera-like illness have also been described [26]. In  
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35 172 several reported studies throughout the world, *Aeromonas* spp. have been isolated at a  
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37 173 rate of 0.6 to 7.2% from patients with diarrhea predominantly from infants and children  
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39 174 [3, 10, 18, 31, 43, 53]. The highest rate, 52.4%, was detected in Peruvian infants with  
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41 175 severe diarrhea although 57.6% of the *Aeromonas*-positive patients had mixed  
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43 176 infections with other enteric pathogens [35]. A similar high frequency of co infection  
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45 177 has been found in Bangladesh and India [3, 43]. Our results are in agreement with those  
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47 178 found in most of the above surveys. Thus, *Aeromonas* spp. was isolated from 4.0% of  
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49 179 the patients, most of them being infants (46.9%) and other pediatric patients (21.9%).  
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51 180 Clinically, acute illness was the most common symptom whereas fever, vomiting or  
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181 chronic diarrhea were present in only a few cases. In addition, common enteric  
182 pathogens other than *Aeromonas spp.* were recovered from more than one third of  
183 diarrheic stools. Because of the ubiquitous nature of *Aeromonas spp.* in raw foods and  
184 water, it has been argued that in mixed infections these bacteria are transient colonizer  
185 without causal relationship with disease, but also that multiple pathogens may act  
186 synergistically to produce diarrhea [3, 35]. Unlike most surveys that have found peaks  
187 of *Aeromonas*-associated gastroenteritis in warmer months, we did not observe seasonal  
188 patterns for isolation.

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

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

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206 that regardless of their source, *A. media* strains are potentially enterotoxigenic. In this  
207 study, two of the *A. media* positive patients had concurrent infection with *Salmonella*.  
208 *A. veronii* biovar *veronii* and *A. bestiarum* have rarely been isolated from humans and  
209 little information is available regarding its occurrence in clinical specimens; however,  
210 two strains of each species were isolated by us as the sole pathogen from two children  
211 and two elderly men, and also from gastroenteritis in Switzerland, India and Brazil [14,  
212 25, 43].

213 The pathogenicity of *Aeromonas* spp. is multifactorial and complex and may  
214 involve the products of a number of different genes acting either alone or in concert [19,  
215 40]. One rational approach to determine whether *Aeromonas* strains have the potential  
216 to be virulent is to screening for an array of virulence-related genes [2, 52]. A potent  
217 virulence factor of *Aeromonas* [strains](#) is the cytotoxic enterotoxin Act, which is closely  
218 related to aerolysin (AerA), both of them being hemolytic, cytotoxic, enterotoxic and  
219 lethal for mice. *Aeromonas* may also produce heat labile (Alt) and heat stable (Ast)  
220 cytotoxic enterotoxins [19]. Sha et al. [41] established a role for the three enterotoxins  
221 in causing gastroenteritis in a mouse model by developing isogenic mutants with the  
222 major contribution from Act, followed by the Alt and Ast. Results from Albert et al. [3]  
223 suggest that the product of both the *alt* and *ast* genes may act synergistically to induce  
224 severe diarrhea.

225 The incidence of the *aerA*, *alt* and *ast* genes in our clinical isolates was 25, 71.9  
226 and 18.8%, respectively, the *alt*<sup>+</sup>/*ast*<sup>+</sup> combination being detected in four of them  
227 (12.5%). These findings are comparable with those of Sinha et al. [43] who, in isolates  
228 from Indian patients with acute diarrhea, found that *act/aerA* accounted for 30%, *alt*  
229 was dominant and *ast* was the less prevalent. Overall, among fecal isolates from Mexico  
230 and Catalonia (Spain), Aguilera-Arreola et al. [2] reported that genes for the aerolysin-

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4 231 hemolysin, Alt, Ast and Alt + Ast toxins accounted for 89.0%, 34.9%, 28.4% and 7.3%,  
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6 232 respectively. Among their *A. caviae* isolates, 90.2% carried the genes encoding cytolytic  
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8 233 activity whereas the *alt* gene was present in only 9.8%. By contrast, we have detected in  
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10 234 *A. caviae* an occurrence of the *aerA* and *alt* genes of 21.7 and 73.9%, respectively. A  
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12 235 survey in Bangladesh [3] found absence of the *act* gene alone and a high incidence of  
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14 236 the *alt* and *alt*<sup>+</sup>/*ast*<sup>+</sup> genes in isolates from children with diarrhea.

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16 237 In addition to the enterotoxic Act/aerA, which causes β-hemolysis, a β-  
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18 238 hemolytic *Vibrio cholerae*-like hemolysin (HlyA) has been described. The *aerA*<sup>+</sup>/*hlyA*<sup>+</sup>  
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20 239 genotype and in some cases the *aerA*<sup>+</sup>/*hlyA*<sup>-</sup> genotype have been reported to be  
21  
22 240 reasonable predictors of human diarrheal disease [24]. Combination of both genes,  
23  
24 241 which are less common in *A. caviae* than in *A. hydrophila*, was only present in the two  
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26 242 *A. hydrophila* isolates. In an earlier study [22], we found that strains belonging to the  
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28 243 genotypes *aerA*<sup>+</sup>/*hlyA*<sup>+</sup>, *aerA*<sup>+</sup>/*hlyA*<sup>-</sup> and *aerA*<sup>-</sup>/*hlyA*<sup>-</sup> were enterotoxic in the suckling  
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30 244 mouse test and had the capability to express other virulence traits (proteolytic,  
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32 245 hemolytic and cytotoxic activities) at different temperatures.

33 246 Swarming motility, a lateral flagella dependent behavior, has been implicated in  
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35 247 biofilms formation and adhesion to eukaryotic cells. The gene cluster *laf* responsible for  
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37 248 *Aeromonas* lateral flagella production [20] was detected in three human isolates (9.4%)  
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39 249 which is lower than the incidence (36.7%) found by Aguilera-Arriola et al. [2].  
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41 250 However, all of our drinking water isolates carried the *laf* gene, two of them (W73 and  
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43 251 W74) being, by PFGE, indistinguishable from the human isolate H13 (Fig 1).

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45 252 In this study, there were three *A. caviae* strains that did not amplify any of the  
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47 253 genes tested although they showed incomplete hemolysis and two were recovered as the  
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49 254 only suspect pathogen. Negative PCR results do not exclude pathogenicity because at  
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51 255 least some *Aeromonas* strains possess a wide range of virulence factors, other than those

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4 256 tested by us, that enable them to overcome host defenses and establish infection. Among  
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6 257 these factors are surface molecules, different toxins, many enzymes, iron acquisition  
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8 258 systems, protein secretion systems and novel virulence determinants that may play an  
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10 259 important role in pathogenesis [19, 39, 40].

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12 260 Our results and those from others suggest that in *Aeromonas* clinical isolates the  
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14 261 distribution pattern of associated-virulence genes varies depending on the species and  
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16 262 geographical location and also that despite current knowledge of *Aeromonas* virulence  
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18 263 determinants, there is still no way to identify a given clinical or environmental isolate  
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20 264 that has the capacity to cause human illness although some data indicate a role of both  
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22 265 the bacterial virulence factors and host-responses [19].

23 266 The presence of TTSS seems to be related to the pathogenicity of some  
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25 267 *Aeromonas* strains [50, 56]. However, it is more frequently found in *A. hydrophila* and  
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27 268 *A. veronii* strains than in *A. caviae* isolates, even from clinical specimens [50]. This fact  
28  
29 269 can explain the lack of detection of TTSS genes in our isolates, as they belong mainly to  
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31 270 the species *A. caviae*.

32  
33 271 Human infections with *Aeromonas* [spp.](#) have occasionally been associated with  
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35 272 hemolytic uremic syndrome (HUS) [17], a life-threatening condition normally  
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37 273 associated with enterohemorrhagic *E. coli* (EHEC) strains producing the cytotoxins Stx1  
38  
39 274 and/or Stx2. The *stx1* gene has been detected in clinical and environmental isolates of *A.*  
40  
41 275 *hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* [23, 44]. As this could explain  
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43 276 reports on HUS related to *Aeromonas* [spp.](#), we investigated the presence of *stx1* and  
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45 277 *stx2* genes and plasmids. None of our isolates were PCR positive for the *stx* genes and  
46  
47 278 28% harbored plasmids. Although most strains of *A. salmonicida* carry at least one large  
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49 279 plasmid, plasmids are detected less commonly (15 to 30%) in human diarrhea-  
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51 280 associated *Aeromonas* isolates [11].  
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281 PFGE, which has remarkable discriminatory power and reproducibility, has been  
282 successfully applied to the subtyping of many pathogenic bacteria [9]. Over the last  
283 years, a number of studies using PFGE and other molecular methods have been used for  
284 comparative typing of clinical and/or environmental *Aeromonas* strains [10, 15, 32, 45-  
285 47] most of them concluding: (i) that specific clones colonized certain aquatic  
286 ecosystems such as drinking water supplies probably as consequence of biofilms  
287 development, (ii) that there was not similarity among clinical isolates, and (iii) that  
288 stools and drinking water isolates were genetically unrelated. In one of the earliest  
289 studies, following a number of cases of diarrhea in children using a small community  
290 water supply, Moyer et al. [32] reported colonization of the water supply with  
291 *Aeromonas* identical ribotypes, but unique ribotypes in *Aeromonas* patient strains,  
292 concluding that water was not the source of *Aeromonas* infections. Similar results were  
293 obtained in French studies using RAPD and PFGE for typing isolates from hospital-  
294 acquired infections and from the hospital water supply [46, 47]. De la Morena et al. [15]  
295 studied *Aeromonas* isolates recovered from children involved in two outbreaks of  
296 gastroenteritis in day-care centers, finding that the PFGE patterns differed in all except  
297 two strains. Also by PFGE, *Aeromonas* isolates from patients with diarrhea and  
298 groundwater isolates from private wells throughout Wisconsin were genetically  
299 unrelated [10]. By using RAPD, REP-PCR and ERIC-PCR, Szczuka and Kaznowski  
300 [45] found that strains isolated from patients in Poland, different parts of Europe and  
301 Asia were different clones and that there was not genetic similarity between clinical and  
302 environmental strains although the persistence of a single clone in a city water  
303 distribution was observed.

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

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

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



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331 **Acknowledgments**

332 This work was supported by Spanish CICYT (grant AGL2004-04672-ALI) and  
333 Junta de Castilla y León (Ayuda Grupo de Excelencia GR155). Manuel Pablos was  
334 beneficiary of a fellowship from MEC.

335 We thank Borja Linage for help in PFGE testing. We also appreciate the kind  
336 collaboration of Dr F. Cachón (Microbiology and Parasitology Service, Hospital of  
337 León).

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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>	<b>ATG AGG TCA TCT GCT CGC GC</b> <b>GGA GCA CAA CCA TGG CTG AT</b>	<b>55</b>	<b>ca 1 Kb</b>	<b>Chacón, 2004</b>
<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) <sup>a</sup>	(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+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) <sup>a</sup>	(2/1)	(0/1)	(1/0)		(1/0)				
						<i>A. caviae</i>	2		
						<i>A. hydrophila</i>	1		

<sup>a</sup>F, 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

