Eight *Aeromonas hydrophila*-like arabinose-negative isolates from diverse sources (*i.e.*, river freshwater, cooling-system water pond, diseased wild European eels, and human stools) sampled in Valencia (Spain) during 2004–2005, were characterized by 16S rRNA gene sequencing and extensive biochemical testing along with reference strains of most *Aeromonas* species. These isolates and all reference strains of *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* showed a 16S rRNA sequence similarity of 99.8–100%, and they all shared an identical phenotype. This matched exactly with that of *A. hydrophila* subsp. *dhakensis* since all strains displayed positive responses to the Voges-Prokauer test and to the use of D-lactate. This is the first report of *A. hydrophila* subsp. *dhakensis* recovered from environmental samples, and further, from its original isolation in India during 1993–1994. This was accurately identified and segregated from other clinical aeromonads (*A. hydrophila* subsp. *hydrophila*, *A. caviae*, *A. veronii* biovars *veronii* and *sobria*, *A. trota*, *A. schuberti* and *A. jandaei*) by using biochemical key tests. The API 20 E profile for all strains included in *A. hydrophila* subsp. *dhakensis* was 7047125. The prevalence of this species in Spanish sources was higher for water (9.4%) than for feces (6%) or eels (1.3%). Isolates recovered as pure cultures from diseased eels were moderately virulent (LD₅₀ of 3.3×10⁶ CFU fish⁻¹) to challenged eels in experimental trials. They were all resistant to ticarcillin, amoxicillin-clavulanic acid, cefoxitin, and imipenem, regardless of its source. Our data point to *A. hydrophila* subsp. *dhakensis* as an emerging pathogen for humans and fish in temperate countries.

Key words: *A. hydrophila* subsp. *dhakensis*, phenotypic profile, 16S rRNA gene sequencing, emerging pathogen.

The genus *Aeromonas* (family *Aeromonadaceae*) is ubiquitous in aquatic ecosystems that include chlorinated drinking water, raw sewage, and natural waters (*i.e.*, fresh and brackish water) and free-living fish in such habitats (27). *Aeromonas* species are most commonly implicated as the causative agents of gastroenteritis in tropical countries, with sepsis a fatal complication of *Aeromonas* infectious diseases, particularly in immunocompromised patients (22). In addition, some *Aeromonas* species are one of the major causative agents of diseases in reared and wild fish (2, 13).

*Aeromonas hydrophila* subsp. *dhakensis* was originally isolated from patients with diarrhea in Dhaka, Bangladesh (India) during 1993–1994 (20, 23). Since then *A. hydrophila* subsp. *dhakensis* has not been isolated in any region of the world, but many reports have focused on its relationship with other *A. hydrophila* subspecies. Thus, depending on the housekeeping genes used (*i.e.*, 16S rRNA, *rpoB*, or *dhaJ*), *A. hydrophila* subsp. *dhakensis* exhibits high divergence from *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae* (31, 32) or it does not (24, 35). In addition, it has been recently reported that type strains of the species *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* clustered together in the phylogenetic tree derived from concatenated *gyrB-rpoD* sequences (31). However, the phenotypic profile of these two arabinose-negative *Aeromonas* species mostly differed (20, 30), as well as their respective genomic homology (DNA-DNA relatedness) with the type strain of the species *A. hydrophila*, which was 46% in the case of *A. aquariorum* (30) but 78 to 84% in the case of *A. hydrophila* subsp. *dhakensis* (20).

Identification of aeromonads by using biochemical schemes is difficult (1) while numerical studies have obtained quite high success showing a good correlation with genotypic identification (42). Most clinical microbiology laboratories still routinely rely on easy-to-use phenotypic methods (39); therefore, key traits for discriminating at least those *Aeromonas* species relevant in clinical sources should be well defined (25, 39). In a previous study, 215 *Aeromonas* isolates were recovered from river water and cooling systems (8), from wild European eels (13), and from human feces, collected during a 1-year period at locations in the river Xúquer floodplain, a highly urbanized and industrialized metropolitan area (753,552 inhabitants) around the city of Valencia (800,469 inhabitants). Eight out of these 215 aeromonads were presumptively identified as *A. hydrophila*, despite their negative production of acid from L-arabinose. The aim of the present study was to fully identify these *A. hydrophila*-like arabinose-negative strains, and to assess their clinical and veterinary relevance. For this purpose we used 16S rRNA gene sequencing and extensive biochemical testing, and also other assays to check for its susceptibility to antimicrobials and its virulence in fish.

**Materials and Methods**

**Bacterial isolates**

Strains ABF132, ABF144, and ABF145 were isolated from a wild European eel caught in the Albufera lake in El Palmar (Valencia, Spain) in November 2004 (13); strains MA17 and MA26 were isolated from two freshwater samples collected in the River Júcar...
in Alberique (Valencia, Spain), in October 2004 and February 2005, respectively (8); and strain MA131 was isolated from the water of a cooling-system pond in Catarroja (Valencia, Spain) in November 2005 (8). Strains 133.341 and 133.343 were isolated from stools on xylose-galactosidase plates (16) at the “Servicio de Microbiología, del Hospital Universitario La Fe” (Valencia, Spain), in September 2005. Strain CECT 4588 was originally recovered from feces of a patient with diarrhea in the Netherlands as isolate AH290 (12). This was identified as “A. hydrophila-like arabinose-negative” by others (42).

**PCR amplification, 16S rRNA sequencing, and phylogenetic analysis**

The almost complete 16S rRNA gene sequence of strains ABF132, ABF144, ABF145, MA17, MA26, MA131, 133.341, 133.343, CECT4588, *A. hydrophila* subsp. *dhakensis* CECT5743, *A. hydrophila* subsp. *dhakensis* CECT5745, *A. aquariorum* MDC310, and *A. aquariorum* MDC317 was obtained by the Colección Española de Cultivos Tipo (CECT) service (Valencia, Spain). Bacterial genomic DNAs were extracted according to a method described previously (37). Universal primers (Invitrogen, Life Technologies) used were 516V (forward) and 599R (reverse) for a stretch around 1,000 nt close to the 5' end (targeting positions for these primers are 8–25 and 1,099–1,113, respectively [*Escherichia coli* numbering]) (5), and P609D (forward) and P1525R (reverse) targeting positions 785–802 and 1,525–1,541, respectively (*Escherichia coli* numbering) (26). PCR mixtures were composed of 5.0 μL PCR buffer (10×), 0.75 μL MgCl₂ (100 mM), 1.0 μL dNTPs (10 mM each), 1.0 μL each forward and reverse primers (50 μM), 0.5 μL Taq polymerase (6 U μL⁻¹; New England Biolabs) and

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**Fig. 1.** Unrooted neighbor-joining phylogenetic tree derived from the 16S rRNA gene sequences of the *Aeromonas* strains used. GenBank accession numbers for 16S rRNA gene sequences obtained in the present study are JQ034990 to JQ034600.
5.0 μL template DNA (50 ng μL⁻¹) in a total volume of 50 μL. PCR amplifications of the DNA templates were performed using a PTC-100 ThermoCycler (MJ Research). The conditions for 16S rRNA gene amplification were (i) 4 min at 94°C; (ii) 30 cycles of 1 min at 94°C, 1 min 30 s at 52°C and 2 min at 72°C; and (iii) a final elongation step of 10 min at 72°C. Amplified products were examined by agarose gel electrophoresis (1.2%) and ethidium bromide staining. Purified amplicons (Mo Bio Laboratories) were sequenced by the dyeoxy method using the BigDye Terminator v. 3.0 Ready Reaction cycle sequencing kit and analyzed in an ABI PRISM 3730 sequencer (Applied Biosystems). Sequencing primers were the same as those used in the amplification reaction but diluted tenfold (5 pmol).

The sequences obtained were aligned by CLUSTAL W program, version 1.83 (41) with the sequences of the type and reference strains of the members of the genus Aeromonas (Fig. 1) that were available in GenBank. Genetic distances and clustering were obtained using Kimura's 2-parameter method. Phylogenetic trees were constructed by the neighbor-joining and maximum-parsimony methods using MEGA4 program (40). Stability of the relationships was assessed by bootstrapping (1,000 replicates).

**Phenotypic characterization**

- Spanish isolates, strain A. hydrophila-like CECT4588, and reference strains of A. hydrophila subsp. dhakensis (CECT7544), CECT7543, and CECT7545), A. aeruvarium (MDC47, MDC310, and MDC317), A. hydrophila subsp. hydrophila (CECT8397), A. veronii bv. veronii (CECT4257), A. veronii bv. sobria (CECT4835), A. sobria (CECT4245), A. jandaei (CECT4228), A. popoffii (CECT4995), A. bestiarum (CECT4227), A. allosaccarophila (CECT4199), A. eucrenophila (CECT4242), A. encheleia (CECT4342), A. trota (CECT4255), A. enteropelogenes (CECT4418), A. caviae (CECT838), A. media (CECT4322), and A. schuberti (CECT4240), and A. dvenera (CECT4254) were examined in 45 tests described by us as valuable traits for identifying Aeromonas (42). In addition, testing of the use of urocanic acid and β-hemolytic activity against sheep blood was performed as reported by others (18, 23). All tests were incubated at 28°C. These data for A. hydrophila subsp. ranae LGM19707, A. fluvialis CECT 7401, A. simiae CIP 107798, A. tecta CECT 7082, A. molluscum CECT 5864, A. bivalvium CECT 7113, A. piscicola CECT 7443, A. taiwanensis CECT7403, A. sanarellii CECT7402, and A. rivuli CECT 7518 were searched in published reports (3, 4, 7, 11, 15, 19, 21, 32, 33). Phenotypes were compared using the Simple Matching and Jaccard's similarity coefficients, and clustering was achieved by the unweighted pair group method using the PUPMA. All analyses were performed using NTSYSSpce version 2.0.

In addition, API 20 E Strips (BioMérieux) were used in all isolates in order to know their API 20 E code.

**Antimicrobial susceptibility assays**

Minimal inhibitory concentrations (MICs) of kanamycin (K), tetracycline (TET), nalidixic (NA), oxolinic (AO) acid, flumequine (UB), erythromycin (ERY), rifampycin (RD) and chloramphenicol (CHL) were determined by the microbroth dilution method (9). In addition, MICs of amoxicilin/clavulanic (AMC), cefotaxime (CTX), cefoxitin (FOX), cefazidime (CAZ), imipenem (IPM), piperacillin (PRL), ticarcillin (TIC), aztreonam (ATM), cefepime (FEP), ciprofloxacin (CIP), netilmicin (NET), norfloxacain (NOR) and levofloxacain (LEF) were determined using E-strips (MICE; Oxoid, Madrid, Spain). Finally, susceptibility to the antibiotics tested was evaluated on the basis of MIC values obtained, in accordance with the breakpoints recommended by the Clinical and Laboratory Standards Institute (9).

**Virulence for European eel**

For the challenge experiment, six young eels of around 10-20 g were challenged by intraperitoneal injection with each of the bacterial doses (10⁶, 10⁵, 10⁴, 10³, 10², 10¹ CFU mL⁻¹), or with 0.1 mL PBS (controls). Each set of six eels was kept in a 20 L aquarium under the following conditions: i) dechlorinated tap water, ii) water temperature around 20°C, iii) oxygen concentration in water above 90% saturation, and iv) fish were not fed. Mortality was recorded daily for 10 days and was only considered if the challenged bacterium was recovered as pure culture from the internal organs.

The bacterial strains used were ABF132, ABF144, and ABF145, which had been isolated by Esteve and Alcaide (13) from a specimen of wild European eel that had suffered from hemorrhagic disease. The primary culture obtained originally from the kidney and liver of this specimen had been pure (i.e., strains ABF132 and ABF144, respectively), but that recovered from the ulcer was not (13). In the latter case we found three types of colonies and, among them, the colony matching ABF154 was the most abundant (personal communication). Bacterial virulence (LD₅₀ expressed as inoculated colony forming units, CFU fish⁻¹) was calculated (38).

**Accession numbers for the 16S rRNA sequence data obtained from GenBank**

EL085557 (A. aeruvarium MDC 47), FJ230076 (A. sanarellii A2-67), FJ230077 (A. taiwanensis A2-50), AJ508765 (A. hydrophila subsp. dhakensis LGM 19562), X60408 (A. caviae NCIMB 13016), X60415 (A. trota ATCC 49657), X60469 (A. hydrophila subsp. ranae LGM 19707), X60410 (A. media ATCC 33907), X60404 (A. hydrophila subsp. hydrophila ATCC 7966), S3922 (A. allosaccarophila CECT 4199), X60412 (A. sobria NCIMB 12065), FJ76000 (A. rivuli WB4-1-19), X60417 (Aeromonas sp. ATCC 35941), A224308 (A. popoffii MG 31754), X60411 (A. eucrenophila NCIMB 74), AU223409 (A. encheleia CECT 4342), AY532690 (A. molluscum 848), X60406 (A. bestiarum CIP 7430), AF134065 (A. salmonicida subsp. pepinoleticia 34me1), X60405 (A. salmonicida subsp. salmonicida NCIMB 1102), X60407 (A. salmonicida subsp. achromogenes NCIMB 1110), AB027544 (A. salmonicida subsp. smithii ATCC 49793), X74680 (A. salmonicida subsp. masoucida ATCC 27013), FM999971 (A. piscicola S1.2), X60413 (A. jandaei ATCC 49568), A170914 (A. cunicola ATCC 3248), FJ230078 (A. fluvialis 717), X60414 (A. veronii bv. veronii ATCC 35624), AJ536821 (A. simiae IBS 6874), X60416 (A. schuberti ATCC 43700), GQ365710 (A. diversa CECT 4254).

**Nucleotide sequence accession numbers**

GenBank accession numbers for 16S rRNA gene sequences obtained in the present study are JQ034588 to JQ034600.

**Results and Discussion**

**Phylogenetic analysis**

The 16S rRNA gene sequence was obtained from eight A. hydrophila-like arabinose-negative isolates, from strain CECT4588, and from A. hydrophila subsp. dhakensis CECT 5743 and CECT 5745, and A. aeruvarium MDC310 and MDC317 (GenBank accession numbers: JQ034588 to JQ034600). These were compared with those of A. hydrophila subsp. dhakensis LGM 19562 and A. aeruvarium MDC 47 and with those from other Aeromonas-type strains (Fig. 1). The 16S rRNA gene sequence similarity among the A. hydrophila-like arabinose-negative Spanish isolates, strain CECT 4588, and the A. hydrophila subsp. dhakensis and A. aeruvarium reference strains was 100–99.8% (0 to 3 bp differences), in accordance with that reported solely for the type strain of these species (31). These sequence similarity values of the 16S rRNA gene were in line with those reported, among strains, for other Aeromonas species (7, 10, 15, 19, 202).
Thus, our phylogenetic results indicated that *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* strains are extremely similar, suggesting that their genomic homology should be checked, especially as experiments of DNA-DNA hybridization between them fail to be performed.

On the other hand, *A. hydrophila*-like arabinose-negative Spanish isolates, strain CECT 4588, and the *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* reference strains belonged to the “*A. caviae*-*A. trota*” branch (Fig. 1) of the *Aeromonas* phylogenetic tree, which also includes the species *A. sanarelli* and *A. taiwanensis* (4). At present, 16S rRNA gene sequencing is widely available in reference laboratories, while biochemical testing is still appropriate for separating those *Aeromonas* species which are phylogenetically close (17). Regarding this finding, the fifteen strains tested by us were clearly segregated from the species *A. caviae* and *A. trota* (Table 1; Fig. 2), as well as from *A. sanarelli* and *A. taiwanensis* (Fig. 2).

### Phenotypic analysis

The *A. hydrophila*-like arabinose-negative isolates, strain *A. hydrophila*-like CECT 4588, and type and reference strains of the species *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* were grouped by numerical analysis at 100% phenotypic similarity (S), using both simple matching (S_{SM}) (Fig. 2) and Jaccard’s (S_{J}) coefficients. Moreover, they were all clearly segregated from the other type strains of the *Aeromonas* species whose phenotypic profile was included in the numerical analysis (Fig. 2). All strains included in the “*A. hydrophila* subsp. *dhakensis*-*A. aquariorum*” cluster (Fig. 2) showed the key responses detailed in Table 1. In addition, they all were positive for: motility; cytochrome-oxidase; Table 1. Key phenotypic profile of the *A. hydrophila* subsp. *dhakensis*-*A. aquariorum* cluster* compared with *Aeromonas* type strains characterized in the study

| Biochemical test            | *A. hydrophila* subsp. *dhakensis*–*A. aquariorum* cluster | *A. caviae* | *A. veronii* bv. *veronii* | *A. bestiarum* | *A. encheleia* | *A. media* | *A. veronii* bv. *sobria* | *A. trota* | *A. popoffii* | *A. sobria* | *A. jandaei* | *A. allosaccharophila* | *A. schuberti* | *A. diversa* |
|-----------------------------|------------------------------------------------------------|-------------|-----------------------------|---------------|--------------|------------|-----------------------------|------------|---------------|-------------|----------------|------------------------|-----------------|------------|
| Hydrolysis of arbutin        | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| aesculin                     | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| SDS                          | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| Gas from D-glucose           | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| Voges-Proskauer              | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| ADH (Moellers’)              | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| LDC (Moellers’)              | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| ODC (Moellers’)              | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| Acid from L-arabinose salcin | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| D-cellobiose                 | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| L-fucose                     | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| L-rhamnose                   | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| Use of DL-Lactate            | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |
| Urocanic acid                | +                                                          | +           | +                           | +             | +            | +          | +                           | +          | +             | +           | +             | +                       | +               | +          |

+, all strains are positive; −, all strains are negative

* Strains included in the cluster are: *A. hydrophila* subsp. *dhakensis* CECT 5744\textsuperscript{7}, CECT 5743 and CECT 5745; isolates ABF132, ABF144, ABF145, MA17, MA26, MA131, 133.341, and 133.343; strain CECT4588; and *A. aquariorum* MDC47\textsuperscript{7}, MDC310, and MDC317.
catalase; gluconate oxidation; O/F metabolism; growth with 0–3% (w/v) NaCl; production of H₂S from cysteine; production of indole from tryptophan; hydrolysis of arbutin, casein, starch, elastin, and gelatin (liquefaction); β-haemolysis of sheep red blood cells; and acid production from D-fructose, D-galactose, maltose, D-mannitol, D-mannose, sucrose, and D-trehalose; however, they all were negative for: Gram staining; production of brown diffusible pigment; susceptibility to O/129 (150 μg) (Oxoid discs); growth with 6% (w/v) NaCl; hydrolysis of urea; and acid production from D-xylose, dulcitol, D-fucose, meso-inositol, melibiose, D-raffinose, D-sorbitol, and D-xylitol.

Phenotypic profile of the “A. hydrophila subsp. dhakensis-A. aquariorum” cluster exactly matched that described for A. hydrophila subsp. dhakensis (20) but differed from that described for A. aquariorum (30). Previous data on the response of A. aquariorum strains to the VP test are confusing; the species was originally described as VP-negative (i.e., in text) and -positive (i.e., in the diagnostic table) simultaneously (30) and hence were reported as VP-positive (7, 34) or VP-negative (4, 15) in subsequent reports. Once again, species and have recently reported the positive responses to both, VP test and other clinical aeromonads, such as A. dhakensis strains (13). In addition, we found that A. aquariorum was originally described as VP-negative (i.e., in the diagnostic table) and positive (i.e., in text) for the utilization of DL-lactate (30), but was later reported as DL-lactate-negative by taxonomic reports (7, 15, 34), mostly based on the diagnostic table (30). We have found that the three A. aquariorum strains assayed by us displayed a clear positive response in the VP semisolids medium (42) and in the Lactate utilization medium (18) respectively, similarly to that shown by Spanish isolates as well as by the reference strains of A. hydrophila subsp. dhakensis (Table 1). It should be noted that our results came from the first extensive phenotypic characterization that compares strains of A. aquariorum and of A. hydrophila subsp. dhakensis, along with other Aeromonas species. Accordingly, other authors have recently reported the positive responses to both, VP test and DL-lactate utilization, for A. aquariorum strains isolated from diverse sources in Western Australia (6).

In line with the present results, A. aquariorum cannot be differentiated from A. hydrophila subsp. hydrophila by the use of DL-lactate, contrary to other reports (14). In fact, the pair A. hydrophila subsp. dhakensis-A. aquariorum, which shared an identical phenotype, was separated in our study from A. hydrophila subsp. hydrophila by its inability to produce acid from L-arabinose and L-fucose, and its use of urocanic acid, and further by its ability to hydrolyze sodium dodecyl sulfate (SDS) (i.e., alkyl sulfatase activity) (Table 1). Finally, we have identified the eight A. hydrophila-like arabinose-negative Spanish isolates, and strain CECT4588, as belonging to the species A. hydrophila subsp. dhakensis, on the basis of the overall results (Table 1; Figs. 1 and 2). The API 20 E profile for all strains included in the “A. hydrophila subsp. dhakensis-A. aquariorum” cluster was 7047125, which previously had shown very little prevalence (i.e., 9%; 8 out of 86), which was obtained from Aeromonas strains (13). In addition, we found that A. hydrophila subsp. dhakensis can be accurately identified and segregated from other clinical aeromonads, such as A. hydrophila subsp. hydrophila, A. caviae, A. veronii biovars veronii and sobria, A. trota, A. schubertii and A. jandaei (25, 39), using biochemical key tests (Table 1).

Incidence and clinical and veterinary relevance of Aeromonas hydrophila subsp. dhakensis in Mediterranean Spain

Aeromonas hydrophila subsp. dhakensis was previously isolated in India (23), and was encountered in Valencia (Mediterranean Spain) during a 1-year study (2004–2005) in which 32 water samples (8), 75 wild European eels (13), and an unknown number of feces from humans suffering acute gastroenteritis were analyzed. Among them, the number of Aeromonas-positive samples was 17 (53.1%) and 20 (26.7%), respectively, with 32 patients suffering from Aeromonas gastroenteritis. The overall prevalence of A. hydrophila subsp. dhakensis in these Aeromonas-positive specimens was of 8.7% (6/69), although it was higher for water (17.7%) than for feces (6.25%) or eels (5%). Thus, we have found a wider distribution of A. hydrophila subsp. dhakensis in Spain in comparison with its unique previous finding in association with patients but not with water or fish (23). It is important to note that such water sources were agricultural ponds linked to the Xúquer river basin, which are used for the irrigation of agricultural products; the counts of Aeromonas in these waters ranged from (10⁴ to 10⁶ CFU mL⁻¹) in winter to (10⁴ to 10⁵ CFU mL⁻¹) in summer (8). In addition, wild European eels are caught in Albufera Lake for commercial purposes and are used for human consumption (13). Hence, these findings could have public health implications because Aeromonas infections might be transmitted through the ingestion of contaminated water or food (i.e., vegetables, fish and shellfish, etc.), or by contact with them (22).

Our clinical A. hydrophila subsp. dhakensis isolates (i.e., 133.341 and 133.343) were recovered from two siblings, a 1-year-old boy and a 6-year-old girl, who had acute gastroenteritis accompanied by bloody stools and high-grade (≥39°C) fever and required antibiotic treatment. The fact that these clinical isolates were rather multi-resistant to antibiotics (Table 2) complicated the management of this Aeromonas-mediated diarrhea, similarly to in other geographical areas (39). Thus, strains 133.341 and 133.343 showed the highest MICs for ticarcillin, piperacillin, amoxicillin/clavulanic, cefoxitin, imipenem, flumequine, nalidix acid, oxolinic acid, and erythromycin (Table 2); however, resistance to ticarcillin, amoxicillin-clavulanic acid, cefoxitin, and imipenem were common in the Spanish isolates of A. hydrophila subsp. dhakensis from any source (i.e., stool, water, fish) (Table 2). Fortunately, the latest generation cephalosporins and the fluoroquinolones had the best inhibitory activity in vitro against A. hydrophila subsp. dhakensis isolates (Table 2), as also was described for other clinical Aeromonas (43).

The three isolates of A. hydrophila subsp. dhakensis (i.e., ABF132, ABF144, and ABF145) which have been recovered from a wild European eel suffering from hemorrhagic septicaemia (13), displayed an LD₅₀ dose of 2.6×10³ to 3.3×10⁴ CFU fish⁻¹ in experimental challenges using healthy eels. These data are the first report on the active role of A. hydrophila subsp. dhakensis as fish pathogen, although others have published that a clinical isolate of this species was moderately virulent for challenged trout (36). Interestingly, A. aquariorum, its closest species, was originally recovered
from imported ornamental fish that showed symptoms of weakness (30).

In summary, species *A. hydrophila* subsp. *dhakensis* can be recovered from natural waters, fish, and clinical specimens in Mediterranean Spain. This result suggests its potential role as a waterborne pathogen for humans and fish in temperate countries. In fact, strain CECT4588 was recovered from feces of a patient with diarrhea in the Netherlands in the eighties. *Aeromonas hydrophila* subsp. *dhakensis* can be identified by biochemical key tests. In the present study the phenotypic profile of *A. hydrophila* subsp. *dhakensis* was also demonstrated by the type and reference strains of *A. aquariorum*. Up to now, species *A. hydrophila* subsp. *dhakensis* as well as its closest species *A. aquariorum* have been reported to be distributed throughout warm countries (6, 14, 23, 30). The present results constitute the first report of *A. hydrophila* subsp. *dhakensis* from a temperate country, suggesting the worldwide distribution of this species.

Acknowledgements

This study has been supported by project CGL2007-60565 from the Ministerio de Ciencia e Innovación (Spain). M.D. Blasco is the recipient of a PhD fellowship from the Spanish Government (Ministerio de Educación y Ciencia). We thank Antonio J. Martínez-Murcia for his technical support with phylogenetic analysis.

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