Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from environmental and clinical samples in Spain.

D. Miñana-Galbis, M. Farfán, J.G. Lorén and M.C. Fusté*.

Dpt. Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Divisió IV, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain.

Running title:

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*Corresponding author:

Dr. Mª Carme Fusté Munné

Dpt. Microbiologia i Parasitologia Sanitàries

Facultat de Farmàcia

Divisió IV

Universitat de Barcelona

Av. Joan XXIII, s/n

08028 Barcelona

SPAIN

Tel.: +34 93 402 44 97.

Fax: +34 93 402 44 98.

e-mail: mcfuste@farmacia.far.ub.es

SUMMARY

Aims: To study the phenotypic characteristics of *Aeromonas* spp. from environmental and clinical samples in Spain, and to cluster these strains by numerical taxonomy.

Methods and Results: A collection of 202 *Aeromonas* strains isolated from bivalve molluscs, water and clinical samples were tested for 64 phenotypic properties. 91% of these isolates were identified at species level. *Aer. caviae* was predominant in bivalve molluscs and *Aer. bestiarum* in freshwater samples. Cluster analyses revealed eight different phena: three containing more than one DNA-DNA hybridization group but including strains that belong to the same phenospecies complex (*Aer. hydrophila*, *Aer. sobria* and *Aer. caviae*), *Aer. encheleia*, *Aer. trota* and three containing unidentified *Aeromonas* strains isolated from bivalve molluscs.

Conclusions: *Aeromonas* spp. are widely distributed in environmental and clinical sources. A selection of 16 of the phenotypical tests chosen allowed the identification of most isolates (91%), although some strains remain unidentified, mainly isolates from bivalve molluscs, suggesting the presence of new *Aeromonas* species. Numerical taxonomy was not in total concordance with the identification of the studied strains.

Significance and Impact of the Study: Numerical taxonomy of *Aeromonas* strains isolated from different sources revealed the presence of potentially pathogenic *Aeromonas* spp, especially in bivalve molluscs, and phena with unidentified strains that suggest new *Aeromonas* species.

Keywords: *Aeromonas*, taxonomy, identification, bivalve molluscs, freshwater, clinical samples.

INTRODUCTION

The genus *Aeromonas* consists of ubiquitous, oxidase-positive, facultatively anaerobic, Gram-negative ϒ-proteobacteria autochthonous to aquatic environments. These bacteria are usual microbiota as well as primary or secondary pathogens of fish, amphibians and other exothermic animals, and some motile species (mainly, *Aer. caviae*, *Aer. hydrophila*, and *Aer. veronii* bv. *sobria*) are opportunistic pathogens of humans (Janda and Abbott 1998).

Since the first DNA-DNA hybridization study performed in *Aeromonas* (Popoff *et al*. 1981) and the description of the genus with four phenospecies (*Aer. caviae*, *Aer. hydrophila*, *Aer. salmonicida*, *Aer. sobria*) in *Bergey's Manual of Systematic Bacteriology* (Popoff 1984), 17 hybridization groups (HGs) have been assigned: *Aer. hydrophila* HG1, *Aer. bestiarum* HG2, *Aer. salmonicida* HG3, *Aer. caviae* HG4, *Aer. media* HG5, *Aer. eucrenophila* HG6, *Aer. sobria* HG7, *Aer. veronii* bv. *sobria* HG8, *Aer. jandaei* HG9, *Aer. veronii* bv. *veronii* HG10, unnamed HG11, *Aer. schubertii* HG12, unnamed HG13 (enteric group 501), *Aer. trota* HG14, *Aer. allosaccharophila* HG15, *Aer. encheleia* HG16, and *Aer. popoffii* HG17 (Allen *et al*. 1983; Farmer *et al*. 1986; Hickman-Brenner *et al*. 1987; Hickman-Brenner *et al*. 1988; Schubert and Hegazi 1988; Kuijper *et al*. 1989; Altwegg *et al*. 1990; Carnahan *et al*. 1991a; Carnahan *et al*. 1991b; Carnahan *et al*. 1991c; Martínez-Murcia *et al*. 1992; Esteve *et al*. 1995; Ali *et al*. 1996; Huys *et al*. 1997).

The sudden description of such a high number of genospecies in *Aeromonas* has meant most laboratories (e.g. clinical) have not discriminated among the species that belongs to the *Aer. hydrophila*, *Aer. caviae* and *Aer. sobria* complex (Janda and Abbott 1998), because of the extensive number of phenotypic tests required for an accurate biochemical identification of all genospecies (Altwegg *et al*. 1990; Abbott *et al*. 1992; Kämpfer and Altwegg 1992; Janda *et al*. 1996; Oakey *et al*. 1996; Carson *et al*. 2001).

Although several numerical studies of *Aeromonas* strains have been performed (Kämpfer and Altwegg 1992; Carnahan and Joseph 1993; Esteve 1995; Noterdaeme 1996; Kaznowski 1997), none of them included isolates from bivalve molluscs.

The present study was undertaken to identify *Aeromonas* strains isolated from different sources and to cluster them by numerical taxonomy.

MATERIALS AND METHODS

Bacterial strains

A total of 220 strains were analyzed. These included 101 *Aeromonas* isolated from six fresh water samples in Spain: 75 from drinking water springs (Montseny 1, Montseny 2 and Hostalets de Balenyà, Barcelona), 15 from a non-drinking water spring (El Preventori, Alcoi, Alacant), 10 from a pond pool (Hostalets de Balenyà, Barcelona) and an isolate from a drinking water distribution system (kindly provided by Dr. Xavier Lizana).

Eighty-eight *Aeromonas* strains were isolated from marine bivalve molluscs of different retail markets in Barcelona (Spain).

Thirteen *Aeromonas* strains were kindly donated from a variety of clinical sources: 11 by Dr. Guillem Prats (Hospital de Sant Pau i la Santa Creu, Barcelona) and 2 by Dra. Conxa Segura (Hospital Duran i Reynals, Barcelona). Finally, 18 type and reference strains from CECT (Spanish Type Culture Collection) were also included (Table 1).

Phenotypic characterization

Each strain was tested for 64 phenotypic properties. Unless otherwise stated, incubations were performed at 25ºC (Oakey *et al*. 1996) and all media contained 1% (w/v) NaCl, provided as such or supplemented at the laboratory (Holt *et al*. 1994).

Oxidase-positive, glucose-fermentative, Gram-negative rods with non-swarming production, no sodium requirements, absence of growth at 9% NaCl and resistance to vibriostatic agent O/129 were presumptively identified as *Aeromonadaceae* and stored in TSB with 20% (v/v) glycerol at –40ºC until further analyses were carried out.

The KOH method (Fluharty and Packard 1967) was used to determine the Gram reaction, although the Gram-staining (Hucker staining method) was performed when results were doubtful. The oxidation-fermentation test was performed in O/F basal medium (Difco) supplemented with 1% (w/v) glucose following Hugh and Leifson (1953).

The following tests were carried out as described elsewhere (Gerhardt *et al*. 1994): cell shape; cytochrome oxidase; catalase activity (methods 1 and 2); swarming motility on TSA and citrate utilization (Simmons' citrate agar, method 1) after 7 days; gas production from D-glucose (method 1) and β-hemolysis of sheep blood (method 1) after 48 h; indole production (method 2), methyl red and Voges-Proskauer (VP) reactions, esculin (but with 0.1% esculin and 0.1% ferric ammonium citrate) and starch hydrolyses after 72 h and DNAse test (method 1) after 4 days.

Motility and hydrogen sulfide production from thiosulfate were tested on SIM medium after 72 h. Motility was verified in overnight cultures in peptone water by microscopic examination when there were doubts. β-galactosidase activity was assessed following the manufacturer's instructions (ONPG discs, Oxoid).

The production of a brown diffusible pigment was visually determined after 7 days on TSA medium; growth at different temperatures was determined on TSB after 15 days at 4°C and after 24 h at 25 °C, 36°C and 45 °C; growth at pH 4.5 and 9.0 was tested on TSB after 48 h; growth on Mc Conkey, AD (m-*Aeromonas* selective agar base of Havelaar) (Havelaar *et al*. 1987) and TCBS media was examined after 48 h. Yellow colonies on AD medium were indicative of acid production from dextrin after 48 h.

The following tests were performed as described elsewhere (MacFaddin 1981): the nitrate reduction test was carried out in nitrate broth after 48 h, the urease test (Rustigian and Stuart's urea broth) after 48 h and the lysine (LDC) and ornithine (ODC) decarboxylases and arginine dehydrolase (ADH) tests in Falkow decarboxylase broth after 4 days. Arbutin hydrolysis was carried out as reported elsewhere (Janda *et al*. 1984).

Salt tolerance test [0, 1, 3, 6 and 9% (w/v) NaCl] was carried out following Twedt (1978) after 72 h. Acid production from 1% (w/v) of the following substrates (Twedt 1984): L-arabinose, D-lactose, D-mannose, D-mannitol, salicin, D-sorbitol, and sucrose was determined after 7 days.

The utilization of substrates as sole carbon sources was studied on M70 medium (Véron 1975; Popoff and Lallier 1984). The following substrates [0.2% (w/v) sugars, 0.1% (w/v)

others] were filter-sterilized: acetate, L-arabinose, L-arginine, L-histidine, L-lactate, D-mannitol. Bacterial growth was examined for 14 days. Hydrogen sulfide from cysteine after 4 days and elastase production after 15 days were made according to Popoff and Lallier (1984). Hydrolysis of xanthine after 15 days was determined following Phillips and Nash (1985).

Susceptibility to the vibriostatic agent O/129 (Oxoid, 150µg) and the following antibiotics (Biomerieux): ampicillin (10µg), cephalothin (30µg), colistin (50µg), polymyxin B (300U), streptomycin (10µg) and tetracyclin (30µg) were tested by the disk diffusion method (Jorgensen *et al*. 1999).

Coding of data and computer analysis

Test results were classified as 1 for positive and 0 for negative. Data were analyzed using the NTSYS^{R} (Numerical Taxonomy and Multivariance Analysis System, version 1.8; Rohlf 1993) program. Similarities were calculated using the S_{SM} (Simple Matching Coefficient) and clustered by UPGMA (Unweighted Pair Group Method with Arithmetic Mean Analysis). Results are expressed in a dendrogram (Fig. 1) and the cophenetic correlation coefficient (*r*) was calculated to validate the clustering of strains (Sneath and Sokal 1973).

Test reproducibility

The test error was evaluated by examining 22 strains in duplicate (10% of the total strains), according to the method suggested by Sneath and Johnson (1972).

RESULTS

Identification and distribution of *Aeromonas* species

All *Aeromonas* strains were positive for: rod morphology, glucose oxidation-fermentation, oxidase, O/129 resistance, nitrate reduction, growth at 0%, 1% and 3% NaCl, growth at pH 9, 25ºC and 36ºC (except *Aer. salmonicida* subsp. *salmonicida* CECT 894^T), growth on AD agar and acid production from mannitol (except *Aer. schubertii* CECT 4240^T), and negative for: Gram reaction, growth at 9% NaCl, swarming motility, urea and xanthin hydrolysis. The results of the remaining tests are shown in Table 2.

A selection of 16 phenotypic tests (marked with an asterisk in Table 2) allowed the identification of HGs described in the genus *Aeromonas*. Most species were differentiated by three or more tests, although there were some exceptions. The species belonging to the *Aer. hydrophila* complex (*Aer. hydrophila* HG1, *Aer. bestiarum* HG2 and *Aer. salmonicida* HG3) were differentiated on the basis of sorbitol fermentation and lactate assimilation. *Aer. eucrenophila* (HG6) could be discriminated from *Aer. caviae* (HG4) by gas production from glucose, and from *Aer. encheleia* (HG16) by LDC and arabinose fermentation. Sucrose fermentation allowed differentiation among *Aer. sobria* (HG7) and *Aer. jandaei* (HG9). *Aer. jandaei* was differentiated from *Aer. popoffii* (HG17) on the basis of LDC and growth on lactate. Finally, arabinose fermentation and starch hydrolysis results were opposite in *Aer. sobria* (HG7) and *Aer. veronii* bv. *sobria* (HG8).

A high percentage (91.1%) of *Aeromonas* isolates were identified at species level. The distribution of *Aeromonas* species varied depending on the sample. In fresh water, all isolates were identified and the predominant species was *Aer. bestiarum* (74.3%), followed by *Aer. caviae* (18.8%) and *Aer. salmonicida* (5%). In bivalve molluscs, the predominant species was *Aer. caviae* (45.5%), followed by *Aer. salmonicida* (22.7%) and *Aer. sobria* (6.8%). In this case, 19.3% of isolates remained unidentified. Clinical isolates were identified as *Aer. caviae* (30.8%), *Aer. hydrophila* (30.8%) and *Aer. sobria* (30.8%), and an isolate could not be identified.

Structure of the dendrogram

Eight clusters, comprising at least two strains each, were defined at 89% S_{SM}, while sixteen strains remained unclustered (Fig. 1).

Phenon I, defined at 89% S_{SM}, contained 113 strains, including two type strains (*Aer. hydrophila* CECT 839^T and *Aer. bestiarum* CECT 4227^T). All strains belonged to *Aer. hydrophila* complex (*Aer. hydrophila, Aer. bestiarum* and *Aer. salmonicida* species), except an *Aeromonas* sp. isolate (strain 51C from carpet-shells). This phenon was divided into 4 subgroups (Fig. 1), and the principal differences among them were sorbitol fermentation and growth on lactate (Table 2). This differentiation allowed us to assign subgroup Ia to *Aer. bestiarum*, Ib and Ic to *Aer. salmonicida*, and Id to *Aer. hydrophila*. The main subgroup, Ia (80 strains), clustered 72 *Aer. bestiarum* strains (including CECT 4227^T), six *Aer. salmonicida* strains, an *Aer. hydrophila* isolate and an isolate belonging to *Aer. hydrophila* complex but that could not be ascribed to any genospecies. Subgroup Ib (17 strains) consisted of 11 *Aer. salmonicida*, five *Aer. bestiarum* and an *Aeromonas* sp. strains. Subgroup Ic included 11 strains, mainly *Aer. salmonicida* strains (8), but also *Aer. hydrophila* (1) and two isolates belonging to *Aer. hydrophila* complex but which could not be ascribed to any genospecies. Finally, subgroup Id clustered five *Aer. hydrophila* strains, including CECT 839^T strain.

Phenon II, defined at 92% S_{SM}, included a large oyster isolate and the type strain CECT 4342^T, both in agreement with the current description of *Aer. encheleia* (Esteve *et al*. 1995), except for the positive response in the lysine decarboxylase test and the use of arginine as sole carbon source.

Phenon III, defined at 89% S_{SM}, contained 13 strains, 11 identified as *Aer. sobria* (including the type strain CECT 4245^T), an *Aeromonas* sp isolate (strain A20Cl from clinical sample) and the type strain *Aer. jandaei* CECT 4228^T (Carnahan *et al*. 1991a). The profile of the *Aer. sobria* strains corresponded to the description of this species (Popoff 1984), except for their assimilation of arginine and histidine, so this phenon was assigned to the *Aer. sobria*

species. All these strains hydrolyzed starch and most of them grew on citrate (10 of 11) but did not produce acid from arabinose (9 of 11).

Between phena III and IV there were strain 899T, isolated from wedge-shells, and the type or reference strains *Aer. veronii* bv. *sobria* CECT 4246, *Aer. allosaccharophila* CECT 4199^T and *Aer. veronii* bv. *veronii* CECT 4257^T. The phenotypic profile of 899T hindered its identification (Table 2). CECT 4246, identified as *Aer. veronii* bv. *sobria*, was positive for the arabinose fermentation test and negative for starch hydrolysis and citrate assimilation. CECT 4199^T (*Aer. allosaccharophila*) was in agreement with its description (Martínez-Murcia *et al*. 1992), except for starch hydrolysis, and CECT 4257^T (*Aer. veronii* bv. *veronii*) with the description of *Aer. veronii* bv. *veronii* (Hickman-Brenner *et al*. 1987).

Phenon IV, defined at 89% S_{SM}, contained 65 strains, including the type strains *Aer. caviae* CECT 838^T and *Aer. eucrenophila* CECT 4224^T. The latter was consistent with the description of *Aer. eucrenophila* (Schubert and Hegazi 1988), except for arbutin hydrolysis. Two strains (963N from razor-shells and 739E from cockles) could not be identified at species level. The remaining strains were identified as *Aer. caviae*, although 40% did not grow on lactate, 11% produced H_2S from cysteine (including the type strains) and 65% were positive for the ADH test. This phenon was assigned to *Aer. caviae* species and was divided into 2 subgroups (Fig. 1), IVa (40 strains) and IVb (25 strains, including both type strains). The main difference among both subgroups was growth on lactate (Table 2). Moreover, strains of phenon IV isolated from molluscs (41 isolates) were placed mainly in subgroup IVa (36) and strains isolated from freshwater (19 isolates) in subgroup IVb (18).

Phenon V, defined at 97% S_{SM}, included two strains: 88M, isolated from mussels, and 709OP, from large oysters. These isolates could not be identified at species level. Phenotypic characteristics of this phenon are shown in Table 2.

Phenon VI, defined at 90% S_{SM}, contained five strains isolated from molluscs (848T and 849T from wedge-shells, 93M from mussels, 431E from cockles and 869N from razor-shells), none of which could be identified at species level. All five strains were negative for indole production (Table 2).

A strain isolated from mussels, 95M, was placed between phena VI and VII. It was not identified and showed atypical biochemical reactions such as growth at 6% NaCl, resistance to tetracyclin and negative results for dextrin fermentation and starch hydrolysis (Table 2).

Phenon VII, defined at 95% S_{SM}, contained two *Aeromonas* sp strains, 665N and 868E isolated from razor-shells and cockles, respectively. These strains grew at 6% NaCl and did not produce acid from mannose (Table 2).

Between phena VII and VIII were seven single strains: three type strains (*V. furnissii* CECT 4203^T, *Aer. media* CECT 4232^T, *Aer. schubertii* CECT 4240^T) and four isolates from molluscs (290T and 912T from wedge-shells, 924O from oysters and 292C from carpet-shells). These isolates were aeromonads of uncertain taxonomic affiliation, except 292C identified as *Aer. caviae* (Table 2). CECT 4232^T was in agreement with the description of *Aer. media* (Allen *et al*. 1983) but it was motile and negative for the ONPG and arginine dehydrolase tests. CECT 4240^T matched the description of *Aer. schubertii* (Hickman-Brenner *et al*. 1988).

Phenon VIII, defined at 97% S_{SM}, included the type strains *Aer. trota* CECT 4255^T and *Aer. enteropelogenes* CECT 4487^T. These strains were identified as *Aer. trota* and were consistent with Carnahan *et al*. (1991b).

Finally, four strains were placed below phenon VIII: A15Cl (*Aer. caviae* strain ampicillin sensitive, isolated from a clinical sample), *V. cholerae* CECT 514^T, *Aer. salmonicida* subsp. *salmonicida* CECT 894^T and *E. coli* CECT 405. CECT 894^T was in agreement with the current description of *Aer. salmonicida* subsp. *salmonicida* (Popoff 1984), except for arabinose and histidine assimilation.

Clustering of strains by numerical taxonomy

The cophenetic correlation coefficient (*r*) between the similarity matrix and the corresponding dendrogram derived from S_{SM} was 0.88. It falls in the range 0.74-0.90 of the most frequently occurring cophenetic correlators reported by Sneath and Sokal (1973).

Test reproducibility

The average probability (*p*) of an erroneus result was 0.67%, calculated from the pooled variance (S^2 = 0.0067) of all the unit characters scored for the duplicate strains. All values of S_i^2 scored below 0.1. These values were acceptable according to Sneath and Johnson's criteria (1972).

DISCUSSION

Distribution of *Aeromonas* species

Aeromonads are detected in a wide range of samples such as fresh waters (Araujo *et al*. 1991; Hänninen and Siitonen 1995; Huys *et al*. 1995; Huys *et al*. 1996; Hänninen *et al*. 1997; Borrell *et al*. 1998; Fiorentini 1998), vegetables (Neyts *et al*. 2000), meats and milk products (Borrell *et al*. 1998; Melas *et al*. 1999), fish (Hänninen *et al*. 1997; Wang and Silva 1999; Neyts *et al*. 2000), shellfish and seawater (Borrell *et al*. 1998; Croci *et al*. 2001), and clinical (Moyer 1987; Kuijper *et al*. 1989; Altwegg *et al*. 1990; Hänninen and Siitonen 1995; Borrell *et al*. 1998). The *Aeromonas* species most frequently isolated from these sources belong to the *Aer. caviae* (*Aer. caviae* HG4, *Aer. media* HG5), *Aer. hydrophila* (*Aer. hydrophila* HG1, *Aer. bestiarum* HG2, *Aer. salmonicida* HG3) and *Aer. sobria* (*Aer. sobria* HG7, *Aer. veronii* bv. *sobria* HG8) complexes.

In this study, a high percentage (91%) of *Aeromonas* isolates were identified at species level. In fresh water *Aer. bestiarum* was the predominant species. *Aer. bestiarum* has also been reported as the most predominant *Aeromonas* species in water samples by Hänninen and Siitonen (1995) and Borrell *et al*. (1998), together with *Aer. salmonicida* by Huys *et al*. (1995) and Hänninen *et al*. (1997). However, *Aer. caviae* was predominant in bivalve molluscs followed by *Aer. salmonicida*, in concordance with Borrell *et al*. (1998). *Aer. caviae* has also been described as the most frequent *Aeromonas* species in clinical samples (Moyer 1987; Kuijper *et al*. 1989; Altwegg *et al*. 1990) and has been associated with sewage and polluted waters (Araujo *et al*. 1991; Fiorentini 1998). In this study, the same percentage of *Aer. caviae*, *Aer. hydrophila* and *Aer. sobria* were obtained from clinical samples.

The highest percentage of unidentified *Aeromonas* isolates was found in bivalve samples (19.3%), where few studies have been performed, suggesting that bivalve molluscs could be a source of new *Aeromonas* species.

***Aeromonas* classification**

The mesophilic *Aeromonas* species were identified with 16 phenotypic tests (Table 2). All these tests, except starch hydrolysis, have also been used for the purpose of identification in other studies (Abbott *et al*. 1992; Martínez-Murcia *et al.* 1992; Esteve *et al*. 1995; Huys *et al.* 1997). Starch hydrolysis was useful in *Aer. sobria – Aer. veronii* bv. *sobria* differentiation.

Rhamnose and sorbitol fermentation, lactate and urocanic acid assimilation and growth at 40.5ºC were useful for the phenotypic differentiation of mesophilic *Aer. hydrophila* complex members (Altwegg *et al*. 1990; Hänninen 1994; Hänninen and Siitonen 1995; Kaznowski 1997; Kaznowski 1998). We used sorbitol fermentation and growth on lactate for the differentiation of strains belonging to this complex. All members of *Aer. hydrophila* complex were included in phenon I. Within this phenon we establish four subgroups in which the different species that constitute this group were clearly separate: 94% of *Aer. bestiarum* strains in phenon Ia, 76% of *Aer. salmonicida* in phena Ib and Ic, and 71% of *Aer. hydrophila* isolates in phenon Id (Fig. 1). The only strain of this complex not included in phenon I was *Aer. salmonicida* subsp. *salmonicida* CECT 894^T, but similar results have also been reported in other taxonomic studies (Esteve 1995; Noterdaeme *et al*. 1996).

It has been described that *Aer. sobria* and *Aer. veronii* bv. *sobria* are phenotypically indistinguishable except for the maximal growth temperature (Hänninen and Siitonen 1995; Huys *et al*. 1996). In our study, two tests, starch hydrolysis and arabinose fermentation, allowed us to separate *Aer. sobria* strains and the *Aer. veronii* bv. *sobria* type strain. In spite of these results, we believe that our isolates should be considered as *Aer. veronii* bv. *sobria*, because *Aer. sobria* has never been described from a clinical origin (Hänninen and Siitonen 1995; Huys *et al*. 1996). In this group, hybridization analyses are probably the only reliable way to adscribe isolates to a concrete species. Together with *Aer. sobria*, phenon III included *Aer. jandaei* CECT 4228^T, a similar result to the one described by Noterdaeme *et al*. (1996), showing the close phenotypic relationship between both species (Janda 1991).

Aer. caviae complex includes *Aer. caviae* (HG4), *Aer. media* (HG5) and *Aer. eucrenophila* (HG6). In addition, some authors (Altwegg *et al*. 1990; Altwegg and Lüthy-Hottenstein 1991;

Kämpfer and Altwegg 1992; Hänninen and Siitonen 1995) separate *Aer. media* in two phenotypic subgroups, the clinical motile (HG5A) and the environmental non-motile (HG5B) isolates, although only the growth on lactate differentiated HG5A from HG4. But growth on lactate is not always a useful test. In our study, *Aer. caviae* isolates clustered in phenon IV, which was divided in two subgroups: IVa, which includes the lactate positive strains and IVb, which clusters the lactate negative, but the last subgroup also includes the type strain *Aer. caviae* CECT 838^{T}. On the other hand, *Aer. media* CECT 4232^{T} was unclustered and distant from phenon IV, similar result to the one reported by Noterdaeme *et al*. (1996).

Further studies are nedeed on unidentified isolates, especially these from phena V, VI and VII, to determine if they are new *Aeromonas* species.

In agreement with previous taxonomic studies (Kämpfer and Altwegg 1992; Carnahan and Joseph 1993; Esteve 1995; Noterdaeme *et al*. 1996; Kaznowski 1997), the results obtained with numerical taxonomy were not in total concordance with the identification of the species studied. Identification based on differences in one or two phenotypic charateristics does not reveal the real environmental diversity and allows mistaken identification when results obtained in different laboratories are compared. The present *Aeromonas* species designation needs reorganizing from a practical point of view because in some cases an HG includes strains phenotypically different (i.e. *Aer. salmonicida* HG3 or *Aer. media* HG5), in others, genotypically identical strains were considered in two HGs because they are phenotypically different (i.e. *Aer. veronii* bv. *sobria* HG8 and *Aer. veronii* bv. *veronii* HG10) and, finally, some strains phenotypically indistinguishables were genotypically ascribed to different HG (i.e. *Aer. sobria* HG7 and *Aer. veronii* bv. *sobria* HG8) (Janda 1991; Janda and Abbott 1998).

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Table 1 Type or reference strains used in this study

Phenon	Taxon	Type or reference strains	Source of isolation
Ia	*Aer. bestiarum*	CECT 4227^T = ATCC 51108^T	fish
Id	*Aer. hydrophila*	CECT 839^T = ATCC 7966^T	tin of milk with fishy odour
II	*Aer. encheleia*	CECT 4342^T = ATCC 51929^T	healthy European eel
III	*Aer. jandaei*	CECT 4228^T = ATCC 49568^T	human stool of patient with diarrhea
III	*Aer. sobria*	CECT 4245^T = ATCC 43979^T	*Cyprinus carpio* (carp)
IVb	*Aer. caviae*	CECT 838^T = ATCC 15468^T	epizootic of young guinea pig
IVb	*Aer. eucrenophila*	CECT 4224^T = ATCC 23309^T	fresh water fish
VIII	*Aer. trota*	CECT 4255^T = ATCC 49657^T	human stool
VIII	*Aer. enteropelogenes*	CECT 4487^T = ATCC 49803^T	human faeces
Unclustered strains:			
	Aer. allosaccharophila	CECT 4199^T = ATCC 51208^T	diseased elver of *Anguilla anguilla* (eel)
	Aer. media	CECT 4232^T = ATCC 33907^T	fish farm effluent
	Aer. salmonicida subsp. *salmonicida*	CECT 894^T = ATCC 33658^T	*Salmo salar* (Atlantic salmon)
	Aer. schubertii	CECT 4240^T = ATCC 43700^T	forehead abscess
	Aer. veronii biovar *sobria*	CECT 4246 = ATCC 9071	infected frog suffering from red-leg disease
	Aer. veronii biovar *veronii*	CECT 4257^T = ATCC 35624^T	sputum of drowning victim
	V. cholerae	CECT 514^T = ATCC 14035^T	human faeces
	V. furnissii	CECT 4203^T = ATCC 35016^T	human faeces
	E. coli	CECT 405 = ATCC 10536	

CECT: Spanish Type Culture Collection; ATCC: American Type Culture Collection; ^{T}: type strain.

Table 2 Phenotypic characteristics of phena and unclustered strains analyzed in this study

Phena	I	Ia	Ib	Ic	Id	II	III	IV	IVa	IVb	V	VI
HG[†]		2	3	3	1	16	7		4	4	-	-
No. of strains	113	80	17	11	5	2	13	65	40	25	2	5
Motility	+					+	+	89	83	+	+	+
Brown soluble pigment*	-					-	-	-			-	20
Catalase	99	+	+	91	+	+	+	+			+	+
Gas from glucose*	99	+	94	+	+	+	+	2	-	4	+	-
ONPG	+					+	+	97	95	+	+	+
Methyl red	68	81	41	36	20	50	31	+			+	+
Voges-Proskauer*	96	+	82	91	+	50	+	-			+	-
Arginine dihydrolase	+					+	+	65	70	56	+	60
Lysine decarboxylase*	+					+	+	2	-	4	+	-
Ornithine decarboxylase*	-					-	-	-			-	-
Production of:												
Indole*	99	+	94	+	+	+	+	98	98	+	+	-
H_2S from thiosulfate	93	99	76	73	+	+	+	42	15	84	50	-
H_2S from L-cysteine*	99	+	94	+	+	+	+	15	3	36	-	-
Growth:												
6% NaCl	1	1	-	-	-	-	-	5	8	-	-	-
4°C	96	+	+	+	20	+	+	95	95	96	+	+
45°C	7	9	-	-	20	+	-	5	5	4	-	-
Mac Conkey	99	99	+	+	+	+	+	+			+	+
TCBS	99	99	+	+	+	+	92	68	53	92	+	80
pH= 4.5	1	1	-	-	-	-	-	-			-	-
Hydrolysis of:												
Arbutin*	+					+	-	95	98	92	+	+
DNA	+					+	+	98	98	+	+	+
Elastin*	82	79	88	91	+	-	-	-			-	-
Erythrocytes	93	99	65	91	+	50	92	17	20	12	-	40
Esculin*	+					+	8	98	98	+	+	+
Starch*	+					+	+	+			+	40
Acid from:												
Arabinose*	98	+	+	+	60	-	23	98	98	+	+	+
Dextrin	99	+	+	91	+	+	+	+			+	40
Lactose	9	-	18	64	-	-	15	75	80	68	+	-
Mannitol*	+					+	+	+			+	+
Mannose	+					+	+	91	85	+	+	+
Salicin	88	89	82	82	+	+	8	94	95	92	+	20
Sorbitol*	25	8	71	91	-	-	-	8	13	-	-	-
Sucrose*	+					+	92	+			+	+
Utilization of:												
Acetate	98	+	94	+	80	50	85	95	98	92	+	80
Arabinose	97	+	+	+	40	-	15	97	95	+	+	+
Arginine	99	+	94	+	+	+	+	+			+	+
Citrate	73	70	82	82	80	-	85	66	83	40	+	+
Histidine	+					+	+	+			+	+
L-Lactate*	8	1	-	27	+	-	8	58	95	-	50	40
Mannitol	+					+	+	98	98	+	+	+
Resistance to:												
Ampicillin	+					+	92	98	98	+	+	80
Cephalothin	+					50	15	92	88	+	+	60
Colistin	58	79	-	18	20	-	8	2	-	4	-	-
Polymyxin B	48	64	-	18	20	-	8	-			-	-
Streptomycin	50	48	82	-	80	-	46	43	33	60	-	-
Tetracyclin	1	-	6	-	-	-	-	3	5	-	-	-

*The 16 basic tests used to identify mesophilic *Aeromonas* species.

[†]The most representative hybridization group of each phena.

+, all strains positive; -, all strains negative; numbers indicate percentage of positive strains.

Table 2 (Contd.)

Phena	VII	VIII	95M	290T	292C	899T	912T	924O	A15Cl
HG[†]	-	14	-	-	-	-	-	-	4
No. of strains	2	2	1	1	1	1	1	1	1
Motility	50	+	+	+	+	+	+	+	-
Brown soluble pigment*	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	-	+	+	+	+
Gas from glucose*	-	+	-	+	-	-	-	-	-
ONPG	+	+	+	+	+	+	+	+	+
Methyl red	+	+	+	+	+	-	+	-	-
Voges-Proskauer*	-	-	-	-	-	+	-	+	-
Arginine dihydrolase	+	+	-	-	+	+	-	+	+
Lysine decarboxylase*	+	+	+	-	-	-	+	-	-
Ornithine decarboxylase*	-	-	-	-	-	-	+	-	-
Production of:									
Indole*	+	+	+	+	+	+	-	+	+
H_2S from thiosulfate	-	+	-	-	-	-	+	-	-
H_2S from L-cysteine*	-	+	-	-	-	-	+	-	-
Growth:									
6% NaCl	+	50	+	+	+	-	+	-	-
4°C	+	+	+	+	+	+	-	+	-
45°C	50	+	+	-	-	-	-	-	-
Mac Conkey	+	+	+	+	+	+	+	+	+
TCBS	+	+	-	-	+	+	+	+	+
pH= 4.5	-	-	-	-	-	-	-	-	-
Hydrolysis of:									
Arbutin*	+	50	+	+	+	+	-	-	+
DNA	+	+	+	+	-	+	-	+	+
Elastin*	-	-	-	-	-	-	-	+	-
Erythrocytes	-	+	-	-	-	-	-	-	-
Esculin*	+	-	+	+	+	+	+	-	+
Starch*	+	-	-	+	+	+	+	+	+
Acid from:									
Arabinose*	+	-	+	+	-	-	-	+	+
Dextrin	+	+	-	+	+	+	+	+	+
Lactose	-	-	-	-	+	-	-	-	+
Mannitol*	+	+	+	+	+	+	+	+	+
Mannose	-	+	+	+	-	+	+	+	+
Salicin	+	-	+	-	+	+	+	-	-
Sorbitol*	-	-	-	-	-	-	-	+	-
Sucrose*	+	-	+	+	+	+	+	+	+
Utilization of:									
Acetate	+	+	+	+	+	-	+	+	-
Arabinose	+	-	+	+	+	-	+	+	-
Arginine	+	+	+	+	+	+	+	-	-
Citrate	+	+	+	+	+	-	+	+	-
Histidine	+	+	+	+	+	+	+	-	+
L-Lactate*	+	+	-	+	+	-	+	+	-
Mannitol	+	+	+	+	+	+	+	+	+
Resistance to:									
Ampicillin	+	-	+	+	-	+	+	+	-
Cephalothin	50	+	+	+	+	+	+	-	-
Colistin	-	-	-	-	-	-	+	-	-
Polymyxin B	-	-	-	-	-	-	+	-	-
Streptomycin	-	+	-	-	-	+	-	-	-
Tetracyclin	-	-	+	-	-	-	-	-	-

*The 16 basic tests used to identify mesophilic *Aeromonas* species.

[†]The most representative hybridization group of each phena.

+, all strains positive; -, all strains negative; numbers indicate percentage of positive strains.

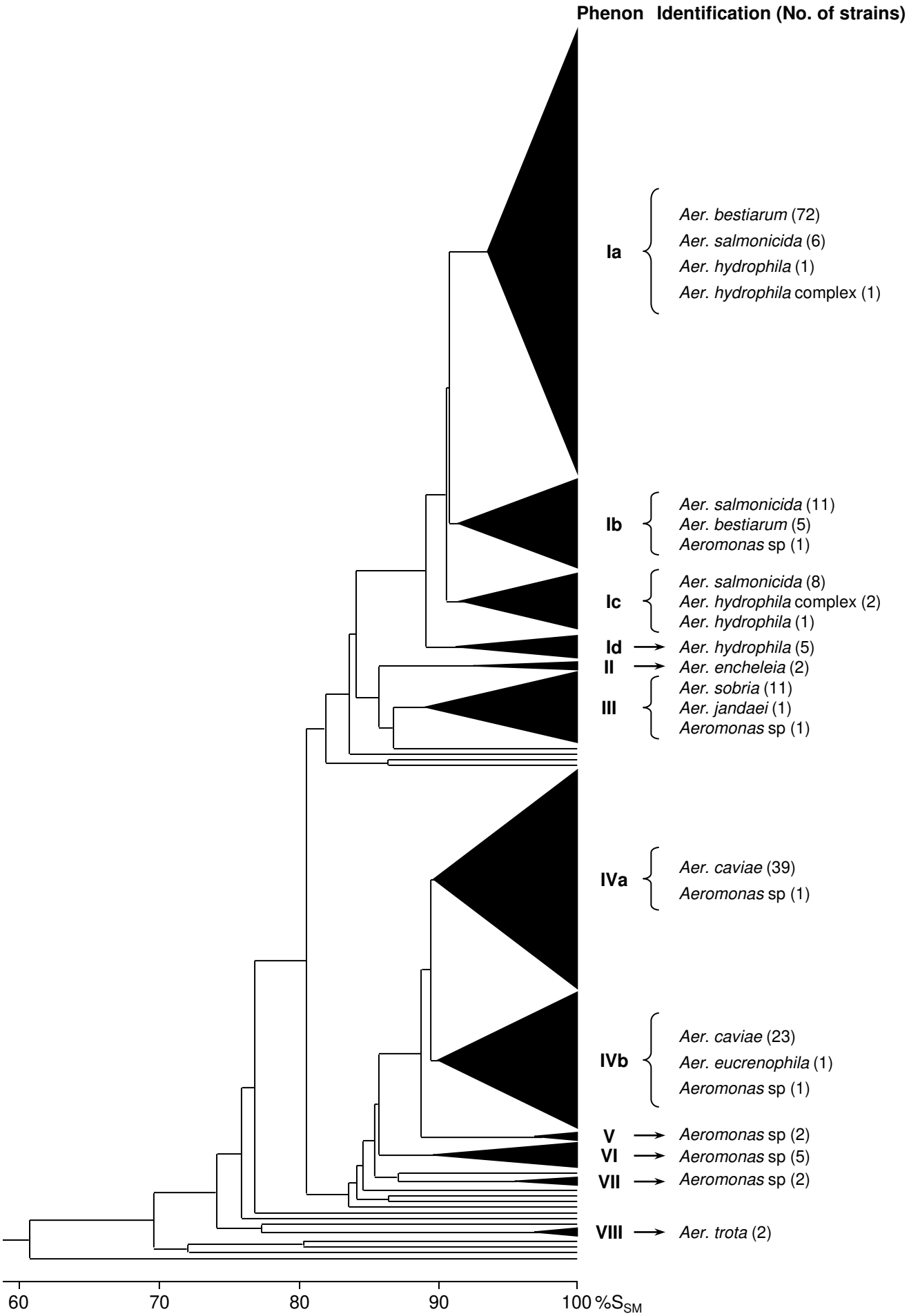

Fig. 1 Simplified dendrogram at 89% S_{SM} using UPGMA