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International Journal of Food Microbiology 84 (2003) 41–49

INTERNATIONAL JOURNAL OF  
Food Microbiology

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## Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico

G. Castro-Escarpulli<sup>a,b</sup>, M.J. Figueras<sup>b,\*</sup>, G. Aguilera-Arreola<sup>a</sup>, L. Soler<sup>b</sup>,  
E. Fernández-Rendón<sup>a</sup>, G.O. Aparicio<sup>a</sup>, J. Guarro<sup>b</sup>, M.R. Chacón<sup>b</sup>

<sup>a</sup>Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, 11340 México Distrito Federal, Mexico

<sup>b</sup>Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, San Llorenç 21, 43201 Reus, Spain

Received 1 May 2002; received in revised form 23 July 2002; accepted 18 August 2002

### Abstract

A total of 82 strains of presumptive *Aeromonas* spp. were identified biochemically and genetically (16S rDNA-RFLP). The strains were isolated from 250 samples of frozen fish (*Tilapia*, *Oreochromis niloticus niloticus*) purchased in local markets in Mexico City. In the present study, we detected the presence of several genes encoding for putative virulence factors and phenotypic activities that may play an important role in bacterial infection. In addition, we studied the antimicrobial patterns of those strains. Molecular identification demonstrated that the prevalent species in frozen fish were *Aeromonas salmonicida* (67.5%) and *Aeromonas bestiarum* (20.9%), accounting for 88.3% of the isolates, while the other strains belonged to the species *Aeromonas veronii* (5.2%), *Aeromonas encheleia* (3.9%) and *Aeromonas hydrophila* (2.6%). Detection by polymerase chain reaction (PCR) of genes encoding putative virulence factors common in *Aeromonas*, such as aerolysin/hemolysin, lipases including the glycerophospholipid-cholesterol acyltransferase (GCAT), serine protease and DNases, revealed that they were all common in these strains. Our results showed that first generation quinolones and second and third generation cephalosporins were the drugs with the best antimicrobial effect against *Aeromonas* spp.

In Mexico, there have been few studies on *Aeromonas* and its putative virulence factors. The present work therefore highlights an important incidence of *Aeromonas* spp., with virulence potential and antimicrobial resistance, isolated from frozen fish intended for human consumption in Mexico City.

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**Keywords:** *Aeromonas*; Mexico; Frozen fish; Virulence factors; Antimicrobial patterns; 16S rRNA-RFLP

### 1. Introduction

*Aeromonas* is an environmental microorganism autochthonous of aquatic environments that can be

sporadically transmitted to humans (Austin and Adams, 1996; Borrell et al., 1998; Janda and Abbott, 1998). Food of animal origin, seafood and vegetables have been considered an important vehicle of *Aeromonas* spp. infections (Altwegg et al., 1991; Kirov 1993; Mattick and Donovan, 1998). Gastroenteritis is the most prevalent human infection caused by *Aeromonas* spp., although other severe illnesses, such as

\* Corresponding author. Tel.: +34-977759321; fax: +34-977759322.

E-mail address: [mjfs@fmcs.urv.es](mailto:mjfs@fmcs.urv.es) (M.J. Figueras).

systemic infections, are less frequent (Janda and Abbott, 1998) and normally associated with immunosuppressed patients (Ko et al., 2000). In addition, these microorganisms are also considered important fish pathogens (Austin and Adams, 1996). A number of putative virulence factors (aerolysin/hemolysin, proteases, lipases, DNases) that may play an important role in the development of disease, either in humans or in fish, have been described in several species of the genus (Howard et al., 1996; Pemberton et al., 1997; Santos et al., 1999; Soler et al., 2002). *Aeromonas* spp. are able to survive and multiply at low temperatures in a variety of food products stored between  $-2$  and  $10$  °C such as beef, roast beef and pork (Krovacek et al., 1992; Mano et al., 2000), and can produce virulence factors even at these low temperatures (Mateos et al., 1993; Merino et al., 1995). Although the number of food-borne outbreaks caused by *Aeromonas* spp. have been quite limited so far (Altwegg et al., 1991; Krovacek et al., 1992; Mattick and Donovan, 1998), the presence of *Aeromonas* spp. in the food chain should not be ignored. Despite the number of surveys on the incidence of *Aeromonas* spp. in food products, there have been few studies on frozen fish. In addition, these studies have used strains which have been inaccurately identified when using traditional biochemical techniques (González et al., 2001), leading to unreliable results (Neyts et al., 2000).

The main objective of this study was to genetically re-identify previously biochemically identified *Aeromonas* spp. isolated from frozen fish intended for human consumption. The species prevalence, putative virulence and antimicrobial susceptibility patterns of the strains isolated were also studied.

## 2. Materials and methods

### 2.1. Bacterial strains and growing conditions

Eighty-two strains of presumptive *Aeromonas* spp. were isolated from 250 frozen fresh water fish (*Tilapia*, *Oreochromis niloticus niloticus*) purchased in local markets in Mexico City. Twenty-five grams of fish flesh were weighed aseptically and homogenized for 2 min in stomacher bags containing 225 ml of alkaline peptone water. After 18 h of incubation at 37

°C, an aliquot of the enrichment was inoculated in blood agar containing 30 mg/l ampicillin and incubated for 24 h at 28 °C. Five presumptive *Aeromonas* colonies were selected for further identification but only one isolate representing a species was considered from each sample for incidence calculation. Stock cultures of each strain were maintained for short periods at room temperature on blood agar base slants and for longer storage they were either frozen at  $-70$  °C in 20% (w/v) glycerol–Todd–Hewitt broth (Oxoid, Madrid, Spain) or lyophilised in 7.5% horse glucose serum as a cryoprotector.

### 2.2. Phenotypic identification

Before each test, all the cultures were grown on tryptose soya agar (TSA) (Oxoid) at 37 °C for 18 h. Strains were first identified as *Aeromonas* spp. at the Medical Bacteriology Laboratory of the National Polytechnic Institute of Mexico City, using the Vitek AutoMicrobic System (Vitek ASM, bioMerieux Vitek, France) and growth 6% sodium chloride was used to discriminate *Aeromonas* from *Vibrio fluvialis* when Vitek was unable to do so. All *Aeromonas* spp. were re-identified biochemically to species level by using 14 tests chosen from those described by Altwegg (1999) and were: indole, gas from glucose, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase by the Moeller's method, esculin hydrolysis, Voges–Proskauer test, acid production from L-arabinose, lactose, sucrose, salicin, *m*-inositol, D-manitol and the  $\beta$ -hemolysine. To identify the biovars of *Aeromonas veronii*, the following tests were used: arginine dihydrolase, ornithine decarboxylase, acid from salicin and esculin hydrolysis.

### 2.3. Molecular identification

All strains were re-identified on the basis of the restriction fragment length polymorphism patterns (RFLP) obtained from the 16S rDNA following the method described by Borrell et al. (1997) and Figueras et al. (2000).

### 2.4. Phenotypic determination of virulence factors

Five colonies of those grown on TSA were suspended in 3 ml of Mueller–Hinton broth (Oxoid). The

density of this suspension was adjusted to 0.5 of the McFarland standard ( $1.5 \times 10^8$  cells  $\text{ml}^{-1}$ ). We added 5 or 10  $\mu\text{l}$  of this suspension to several media or substrates for the phenotypic determination of virulence factors. All strains were tested in duplicate, and when results were different, a third experiment was carried out to resolve the discrepancies.

#### 2.4.1. Hemolytic activity

The strains were tested for  $\beta$ -hemolytic activity on agar base (Oxoid) supplemented with 5% sheep erythrocytes. Five microliters of each suspension was streaked onto the plates and incubated at 22 and 37 °C for 24 h. The presence of a clear colourless zone surrounding the colonies indicated  $\beta$ -hemolytic activity (Gerhardt et al., 1981).

#### 2.4.2. Proteolytic activity

Casein hydrolysis was tested on Mueller–Hinton agar (Oxoid) containing 10% (w/v) skimmed milk (Difco, Barcelona, Spain) by streaking 10  $\mu\text{l}$  of each suspension onto the plates and incubating at 37 °C for 24 h. The presence of a transparent zone around the colonies indicated caseinase activity. Gelatinase was assayed by a radial diffusion method, as described by Gudmundsdóttir (1996), using 3% gelatine (Oxoid) in 1% agarose gel. Ten microliters of each suspension was placed in 4-mm-diameter wells cut into an agarose gel and incubated at 22 °C for 20 h. Then, the plates were immersed in a saturated ammonium sulphate solution at 70 °C to precipitate unhydrolysed gelatine. The presence of a transparent zone around the colonies indicated gelatinase activity.

#### 2.4.3. Lipolytic activity

Lipase activity was determined by three different methods: (i) 10  $\mu\text{l}$  of each suspension was placed into wells cut into 1% (w/v) agarose in phosphate buffer saline containing 1% L- $\alpha$ -lecithin (Sigma, Barcelona, Spain) and incubated at 37 °C for 48 h (Lee and Ellis, 1990); (ii) 10  $\mu\text{l}$  of each suspension was streaked onto plates containing 0.5% tributyrin (Panreac, Barcelona, Spain) emulsified with 0.2% Triton X-100 and incubated at 37 °C for 24 h (Anguita et al., 1993). In both methods, the presence of a transparent zone around the colonies indicated lipase activity; (iii) 5  $\mu\text{l}$  of the suspension was streaked onto resazurine–butter agar and incubated at 37 °C for 24 h; the presence of pink

colonies indicated lipase activity (Amador-López et al., 1993).

#### 2.4.4. Nuclease activity

Extracellular nucleases (DNases) were determined on DNase agar plates (Difco) with 0.005% methyl green. Five microliters of each suspension was streaked onto the plates and incubated at 37 °C for 24 h. A pink halo around the colonies indicated nuclease activity.

#### 2.4.5. Congo red dye uptake

The ability to take up Congo red dye was determined on agar plates supplemented with 50  $\mu\text{g}/\text{ml}$  of Congo red dye. Five microliters of each suspension was streaked onto the plates and incubated at 37 °C for 24 h. Orange colonies were considered positive, different intensities in the dye uptake were expressed as + and ++ (Paniagua et al., 1990).

#### 2.4.6. HEp-2 cell adherence assay

The HEp-2 cell adherence assay was performed as described by Cravioto et al. (1979) and Carrello et al. (1988). All strains were tested in duplicate. The following strains were included as positive controls: *Escherichia coli* E1550 (aggregative adherence AA), *E. coli* E23 (localise adherence AL), *E. coli* E4976 (diffuse adherence AD), *Aeromonas veronii* bv. sobria BC 88 and *Aeromonas caviae* CA25 (aggregative adherence) (Brown et al., 1997; Kirov et al., 2000).

### 2.5. Genetic detection of virulence genes

The presence of genes encoding for aerolysin/hemolysin, extracellular lipases (*lip*, *lipH3*, *pla*, *plc* and the GCAT); serine protease and DNase was determined by polymerase chain reaction (PCR) using primers and conditions already published (Soler et al., 2002).

### 2.6. Antimicrobial susceptibility

The resistance of all strains to different antimicrobial agents was determined by the disk diffusion method (Becton Dickinson, Microbiology Systems, MD, USA) as described (NCCLS, 1999). The antibiotics and concentration ranges tested were as follows: amikacin (30  $\mu\text{g}$ ), nalidixic acid (30  $\mu\text{g}$ ),

Table 1

Comparison of the biochemical and genetic identification (16S rDNA-RFLP) of 82 presumptive *Aeromonas* spp. isolated from frozen fish

| Genetic identification  | Biochemical identification              |  |                                     |  |   |
|---|---|--|-------------------------------------|--|---|
|   | <i>A. hydrophila</i> ,<br><i>n</i> = 17 | <i>A. salmonicida</i> ,<br><i>n</i> = 29 | <i>A. caviae</i> ,<br><i>n</i> = 14 | <i>A. veronii</i> bv. <i>sobria</i> ,<br><i>n</i> = 11 | <i>A. eucrenophila</i> ,<br><i>n</i> = 11 |
| <i>A. hydrophila</i> , <i>n</i> = 2                             | 1                                       |  |                                     |  | 1   |
| <i>A. salmonicida</i> , <i>n</i> = 52                           | 11                                      | 19                                       | 9                                   | 6  | 7   |
| <i>A. veronii</i> bv. <i>sobria</i> , <sup>a</sup> <i>n</i> = 4 | 1                                       | 1  |                                     | 2  |   |
| <i>A. bestiarum</i> , <i>n</i> = 16                             | 4                                       | 7  | 2                                   | 3  |   |
| <i>A. encheleia</i> , <i>n</i> = 3                              |   |  |                                     |  | 3   |
| Other genera, <i>n</i> = 5                                      |   | 2  | 3                                   |  |   |

<sup>a</sup> Strains identified by 16S rDNA-RFLP as *A. veronii* were further identified biochemically to determine the biotypes.

ampicillin (10 µg), carbenicillin (100 µg), cephalothin (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), streptomycin (10 µg), gentamicin (10 µg), imipenem (10 µg), kanamycin (30 µg), neomycin (30 µg), nitrofurantoin (100 µg), penicillin (10 U), piperacillin (100 µg), polymyxin B (300 U), rifampicin (5 µg), tetracycline (30 µg) and trimethoprim–sulfamethoxazol (25 µg). The resistance breakpoints were those defined by the National Committee for Clinical Laboratory Standards (NCCLS, 1999) for Gram-negative bacteria. *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Aeromonas hydrophila* (ATCC 7966<sup>T</sup>), *Aeromonas sobria* (ATCC 42979<sup>T</sup>) and *A. caviae* (ATCC 15468<sup>T</sup>) were used as controls.

### 3. Results

The 82 strains were identified by Vitek as: *A. hydrophila* (46), *A. caviae* (25) and *A. veronii* (11). The same strains were reidentified by 14 biochemical

tests chosen from those described by [Altwegg \(1999\)](#) and the distribution of species was the following: 17 *A. hydrophila*, 29 *Aeromonas salmonicida*, 14 *A. caviae*, 11 *A. veronii* bv. *sobria* and 11 *Aeromonas eucrenophila* (Table 1). After genetic identification, the distribution of species obtained was: 52 *A. salmonicida*, 16 *Aeromonas bestiarum*, 4 *A. veronii* bv. *sobria*, 2 *A. hydrophila*, 3 *Aeromonas encheleia* and five strains had RFLP patterns of other genera (Table 1).

Results of the detection by PCR of the selected genes encoding putative virulence factors and their related phenotypic activity are shown in Table 2. The glycerophospholipid-cholesterol acyltransferase (GCAT) gene was present in all the strains. The genes for aerolysin/hemolysin, lipases, serine protease, and DNase were present in 96%, 97%, 96%, and 90% of the strains, respectively. All strains showed lipase activity with the three methods employed. However, protease activity was different when evaluated with the caseinase test (61%) than with gelatinase (96%). All strains were positive for uptake of Congo red, although with a different intensity, as 45.1% of them produced orange colonies and 54.9%

Table 2

Incidence of virulence genes and phenotypic expression (%) in *Aeromonas* spp. isolated from frozen fish

| Identification<br>16S rDNA-RFLP                    | Aerolysin/<br>hemolysin<br>genes | β-Hemolysis<br>sheep blood          |       | GCAT<br>genes | Lipases <i>lip</i> ,<br><i>lipH3</i> , <i>pla</i> ,<br><i>plc</i> genes | Lipase<br>test <sup>a</sup> | Serine<br>protease<br>genes | Caseinase<br>test | Gelatinase<br>test | DNase<br>genes | DNase<br>test |
|--|----------------------------------|-------------------------------------|-------|---------------|---|-----------------------------|-----------------------------|-------------------|--------------------|----------------|---------------|
|  |                                  | 22 °C                               | 37 °C |               |   |                             |                             |                   |                    |                |               |
|  |                                  | <i>A. hydrophila</i> , <i>n</i> = 2 | 100   |               |   |                             |                             |                   |                    |                |               |
| <i>A. bestiarum</i> , <i>n</i> = 16                | 87                               | 93                                  | 93    | 100           | 100   | 100                         | 100                         | 87                | 100                | 93             | 100           |
| <i>A. salmonicida</i> , <i>n</i> = 52              | 98                               | 94                                  | 94    | 100           | 98  | 100                         | 100                         | 50                | 98                 | 84             | 100           |
| <i>A. veronii</i> bv. <i>sobria</i> , <i>n</i> = 4 | 100                              | 100                                 | 100   | 100           | 75  | 100                         | 50                          | 100               | 100                | 75             | 100           |
| <i>A. encheleia</i> , <i>n</i> = 3                 | 100                              | 0                                   | 0     | 100           | 100   | 100                         | 100                         | 66                | 66                 | 100            | 100           |
| All the strains                                    | 96                               | 90                                  | 90    | 100           | 97  | 100                         | 96                          | 61                | 96                 | 90             | 100           |

<sup>a</sup> Equal response was found with the three methods (1% L-α-lecithin, 0.5% tributyrin and resarsurine–butter agar).

Table 3  
Percentage antimicrobial resistance of *Aeromonas* spp. isolated from frozen fish

| Antibiotic                   | <i>A. hydrophila</i> ,<br>n = 2 | <i>A. salmonicida</i> ,<br>n = 52 | <i>A. bestiarum</i> ,<br>n = 16 | <i>A. veronii</i> bv. sobria,<br>n = 4 | <i>A. encheleia</i> ,<br>n = 3 | Resistant<br>strains (%) |
|------------------------------|---------------------------------|-----------------------------------|---------------------------------|--|--------------------------------|--------------------------|
| Ampicillin                   | 100                             | 100                               | 100                             | 100                                    | 100                            | 100                      |
| Penicillin                   | 100                             | 100                               | 100                             | 100                                    | 100                            | 100                      |
| Piperacillin                 | 0                               | 15.3                              | 25                              | 75                                     | 0                              | 19.4                     |
| Carbenicillin                | 100                             | 100                               | 100                             | 100                                    | 100                            | 100                      |
| Imipenem                     | 50                              | 3.8                               | 19                              | 50                                     | 0                              | 10.3                     |
| Cephalothin                  | 100                             | 100                               | 100                             | 100                                    | 100                            | 100                      |
| Cefotaxime                   | 0                               | 0                                 | 0                               | 0                                      | 0                              | 0                        |
| Cefuroxime                   | 0                               | 0                                 | 0                               | 0                                      | 0                              | 0                        |
| Amikacin                     | 0                               | 23                                | 31                              | 25                                     | 0                              | 23                       |
| Streptomycin                 | 100                             | 71                                | 69                              | 100                                    | 100                            | 75.3                     |
| Gentamicin                   | 0                               | 69                                | 38                              | 75                                     | 0                              | 58.4                     |
| Kanamycin                    | 0                               | 34.5                              | 19                              | 25                                     | 0                              | 28.5                     |
| Erythromycin                 | 50                              | 57.6                              | 44                              | 25                                     | 100                            | 54.5                     |
| Nalidixic acid               | 0                               | 0                                 | 0                               | 0                                      | 0                              | 0                        |
| Ciprofloxacin                | 0                               | 50                                | 25                              | 50                                     | 0                              | 41.5                     |
| Clindamycin                  | 100                             | 100                               | 100                             | 100                                    | 100                            | 100                      |
| Chloramphenicol              | 0                               | 7.6                               | 18.7                            | 0                                      | 0                              | 9                        |
| Neomycin                     | 0                               | 46                                | 44                              | 0                                      | 0                              | 40.2                     |
| Nitrofurantoin               | 0                               | 0                                 | 0                               | 0                                      | 0                              | 0                        |
| Polymyxin B                  | 100                             | 86.4                              | 81                              | 75                                     | 100                            | 85.7                     |
| Rifampicin                   | 100                             | 48                                | 75                              | 50                                     | 100                            | 57.1                     |
| Tetracycline                 | 0                               | 57.6                              | 56                              | 50                                     | 67                             | 44.1                     |
| Trimethoprim–sulfamethoxazol | 50                              | 46                                | 63                              | 25                                     | 67                             | 49.3                     |

reddish orange colonies. All strains showed clear adherence to HEP-2 cells in a typically aggregative pattern (AA).

The resistance patterns obtained with the 77 *Aeromonas* strains against 23 antibiotics are shown in Table 3. All strains showed 100% of resistance to ampicillin, carbenicillin, cephalothin, clindamycin, and penicillin. In addition, the highest resistances encountered were 85.7% to polymyxin B, 75.3% to streptomycin, 58.4% to gentamicin, 57.1% to rifampicin, 54.5% to erythromycin, while the rest were under 50%. In contrast, all the strains were susceptible to nalidixic acid, cefotaxime, cefuroxime and nitrofurantoin.

#### 4. Discussion

Molecular identification by the 16S rDNA-RFLP method (Borrell et al., 1997; Figueras et al., 2000) demonstrated that the most prevalent species in frozen fish were *A. salmonicida* (67.5%) and *A. bestiarum* (20.8%), accounting for 88.3% of the isolates, while

the other strains belonged to the species *A. veronii* (5.2%), *A. encheleia* (3.9%) and *A. hydrophila* (2.6%). Hänninen et al. (1997) also reported *A. salmonicida* and *A. bestiarum* as the most prevalent species in fish.

There were discrepancies on the identification results from the 14 tests selected from those proposed by Altwegg (1999) when compared with those obtained with the genetic method because none of the *A. caviae* strains identified corresponded to this species. In fact, of the 82 strain, only 77 belonged to *Aeromonas* spp. and only 28.5% of strains were correctly identified biochemically (1/2 strains of *A. hydrophila*, 2/4 *A. veronii* and 19/52 *A. salmonicida*). These data highlight the discrepancies between biochemical and molecular methods, although the former methods are very inaccurate, as stated by other authors (Borrell et al., 1997).

*A. salmonicida* has been classically considered a fish pathogen able to develop furunculosis in salmonids, eels, rainbow trout and trout (Austin and Austin, 1993; Austin and Adams, 1996; Sørum et al., 2000; Garduño et al., 2000), but it has also been isolated from other varieties of healthy fish (Frerichs et al.,

1992; Wiklund and Dalsgaard, 1998). This species includes psychrophilic nonmotile bacteria as well as mesophilic bacteria (Janda et al., 1996). All *A. salmonicida* strains investigated were isolated at 37 °C and 79% of them were motile (data not shown). Other species such as *A. hydrophila*, *A. veronii* bv. *sobria*, *A. bestiarum* and *Aeromonas jandaei* have been described in other studies (González et al., 2001). The isolates used in this study were obtained from apparently healthy fresh water fish sold frozen in markets in Mexico D.F. It has been suggested that the fish either harbours the pathogen at the time of capture or become infected during transport (Wiklund and Dalsgaard, 1998). The possibility of spoilage by *Aeromonas* was confirmed by González et al. (2001) in iced wild freshwater fish. Studies on apparently healthy fish have shown that *A. hydrophila* and *A. sobria* are the most frequently isolated species (Santos et al., 1988) in contrast to our findings. The explanation for this disagreement could be that many studies rely on biochemical identification schemes which are not as accurate as genetic identification systems (Borrell et al., 1997; Figueras et al. 2000). Since most studies on *Aeromonas* in fish use these phenotypic techniques, data available on the prevalence of the species are not useful for comparison (Neyts et al., 2000).

The enteropathogenicity of *Aeromonas* spp. has been attributed to the production of exoenzymes, exotoxins and adhesins, although the importance and exact mechanism of each factor associated to the virulence has not been well established (Pemberton et al., 1997). Our results showed that the genes encoding aerolysin/hemolysin, serine protease, extracellular lipases (*lip*, *lipH3*, *pla*, *plc* and GCAT) and extracellular nucleases in strains of frozen fish were highly prevalent, and in addition, practically all the strains showed phenotypic activity. The species *A. bestiarum* and *A. salmonicida* had a high occurrence of aerolysin/hemolysin genes, and the strains were also found to be  $\beta$ -hemolytic. The presence of the mentioned genes in these species has already been reported by other authors (Kingombe et al. 1999; Chacón et al., submitted for publication). The three strains of *A. encheleia* tested did not show  $\beta$ -hemolytic activity but were PCR positive for aerolysin/hemolysin gene; these data is in agreement with those obtained by Chacón et al. (submitted for publication).

The lipases are considered important for bacterial nutrition (Pemberton et al., 1997), although they also have a role in virulence since insertion mutants for the lipase gene *plc* reduces the lethal dose (LD<sub>50</sub>) in mice and fish (Merino et al., 1999). In the present study, the *A. veronii* bv. *sobria* was the species with the least presence of lipase genes (75%). Lipolytic activity evaluated either with resazurine–butter medium,  $\alpha$ -lecithin and tributyrin test showed an equal response for all strains. Thereafter, we recommend the latter method because it is easier to perform and to interpret.

The proteolytic activity of *A. hydrophila* has been correlated with its ability to induce pathology in fish (Austin and Adams, 1996). In the present study, the proteolytic activity of the isolates was evaluated by determining caseinase and gelatinase production. We found similar percentage of serine protease genes (97%) and gelatinase activity (96%), although only 61% of the strains produced caseinase. The caseinase test seemed to be the least useful for evaluating protease activity and subsequently we recommended the use of gelatinase instead.

The DNase genes were detected in 83% of the strains, although DNase activity was observed in all the strains tested. This may indicate that there may be other DNase genes not targeted by our primers that may be responsible for the activity.

The virulence of several enteropathogenic bacteria such as *Yersinia enterocolitica* (Farmer et al., 1992), *Edwardsiella tarda* (Ling et al., 2000), *Vibrio cholerae* (Nataro and Kaper, 1998), enteroinvasive *E. coli* and *Shigella flexneri* (Rico-Martinez, 1995; Nataro and Kaper, 1998) has been correlated with the ability of the isolates to take up Congo red dye. Ishiguro et al. (1985) suggested that this test may also be a good virulence marker of *A. salmonicida*, although this has been questioned by other authors (Ellis et al., 1988). All strains studied showed the ability for binding Congo red in agreement with data reported by other authors in *Aeromonas* (Statner and George, 1987; Palumbo et al., 1989; Paniagua et al., 1990). It has been suggested that siderophores produced by *Aeromonas* spp. could be responsible for the uptake of Congo red (Santos et al., 1999).

In our study, all strains, independent of the species, showed an aggregative type of adherence to HEp-2 cells, in agreement with that described for *A. caviae* (Thornley et al., 1994). It has been reported that this

phenomenon could be due to the presence of type IV pilus adhesins (Thornley et al., 1994; Kirov et al., 2000). A diffused and localised type of adherence on HEp-2 cells was reported for *A. hydrophila* (Bartkova and Ciznar, 1994).

Most strains were 100% resistant to  $\beta$ -lactam antibiotics, with the exception of imipenem (10.3%), piperacillin (19.4%) and second and third generation cephalosporins (cefuroxime and cefotaxime). The strains also showed 100% sensitivity to first generation quinolones (nalidixic acid) and nitrofurantoin. In general, our results are consistent with data reported by other authors for clinical strains (Morita et al., 1994; Ko et al., 1996). Dalsgaard et al. (1994) found that strains of *A. salmonicida* isolated from different geographical areas were 100% susceptible to ampicillin, cephalotin, chloramphenicol, neomycin, polymyxin B and rifampicin. We found that strains of *A. salmonicida* showed a different degree of resistance to these antibiotics, ranging from 7.6% to 100%. It is worth mentioning that susceptibility to ampicillin described by these authors is not common in *Aeromonas*, with the exception of *Aeromonas trola* (Carnahan et al., 1991). Strains of *Aeromonas* isolated from rivers (Goñi-Urriza et al., 2000) showed 59% resistance against nalidixic acid while we encountered no resistance at all (0%). In addition, these authors found 14% resistance to tetracycline and 1% to gentamicin against 44% and 53.4%, respectively, in our strains. A 100% sensitivity to amikacin, ciprofloxacin, chloramphenicol, gentamicin, imipenem and kanamycin was found in a study that included a broad number of clinical and environmental strains (Kämpfer et al., 1999), in contrast to the resistance to ciprofloxacin and gentamicin found here. Also in a previous study analyzing 43 clinical strains of *A. hydrophila*, *A. caviae* and *A. veronii*, between 93% and 100% of them were susceptible to gentamicin and 100% of them to ciprofloxacin (Vila et al., 2002) against 58.4% and 41.5% found in our strains, respectively. Our results show that the first generation quinolones, second and third generation cephalosporins are the drugs with the best antimicrobial effect against *Aeromonas* spp., in agreement with data reported by other authors (Motyl et al., 1985; Ko et al., 1996; Kämpfer et al., 1999; Vila et al., 2002). Therefore, these drugs could be the treatment of choice for extraintestinal infections, and also for

patients with chronic diarrhoea caused by *Aeromonas* spp. including *A. salmonicida* and *A. bestiarum*. These species, although normally considered environmental, have also been involved in human clinical infections (Figueras et al., 1999), although their antimicrobial pattern in genetically identified strains was unknown.

The present work highlights an important incidence of *Aeromonas* spp. in frozen fish intended for human consumption in Mexico City. The high presence of virulence factors and antimicrobial resistance detected in these strains should not be underestimated. In conclusion, frozen fish, such as the one studied here which is consumed raw, may be an important reservoir for *Aeromonas* spp., and be of public health significance.

### Acknowledgements

This work has been supported by: CGPI 200468 and CGPI 990290 from the Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, México; FIS 99/0944 and FIS 96/0579 from Spanish Ministry of Health; from CIRIT (SGR 1999/00103) and from the Fundació Ciència i Salut. We would like to thank: Graciela Margarita Lugo from the Veterinary Microbiology Laboratory, Escuela Nacional de Ciencias Biológicas (México); Dr. S. Kirov from University of Tasmania Clinical School (Australia); Dr. M. Janda from the Department of Health Service Berkeley (California) and Colección Española de Cultivos Tipo (CECT) for kindly providing isolates.

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