

Molecular and phenotypic characterization of *A. hydrophila*-like HG3 strain isolated of an infant with diarrhea in Mexico

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ABSTRACT

A. hydrophila-like HG3 strain was the only enteric pathogen isolated from loose stools of a one-year-old infant from Acaxochitlán Hidalgo, Mexico. Isolation of the HG3 strain (phenotype *A. hydrophila*) from feces of patients with diarrhea has a low worldwide isolation frequency and this is the first report for Mexico. The strain was identified based on biochemical characteristics, RFLP 16S rDNA profile, and 16S rRNA gene sequence. The strain was characterized according to its ability to produce several virulence-related proteins and genes. Aerolysin/hemolysin, heat labile cytotoxic enterotoxin (alt), lipases, including the glycerophospholipid-cholesterol acyltransferase (GCAT), and DNases were phenotypically and genetically detected. The heat-stable cytotoxic enterotoxin (ast) and laf genes were not detected and the strain showed susceptibility to cephalosporin and quinolones. The virulence potential of this particular strain was documented and the clinical importance of similar strains must be made known. The apparently low frequency of the HG 3 strains should not be underestimated because those virulence factors are shared with other *Aeromonas* species that exhibit higher incidences. The present study adds new information to the knowledge on these particular strains.

RESUMEN

Se aisló una cepa de *Aeromonas hydrophila* perteneciente al grupo de hibridación 3 (HG3) como único patógeno en una muestra de heces diarreicas proveniente de un niño de 1 año de edad que habita en el poblado de Acaxochitlán, Hidalgo. A nivel mundial, el aislamiento de cepas del HG3 (fenotipo *A. hydrophila*) a partir de heces diarreicas se reporta con una muy baja incidencia y el aislamiento que reportamos en el presente artículo es el primer reporte en México. La cepa se identificó por pruebas bioquímicas y por métodos genéticos (RFLP-16S rDNA y por secuenciación del 16S rDNA). Adicionalmente, se determinó su capacidad de producir diversas enzimas extracelulares relacionadas con la virulencia y se detectaron, vía PCR, los genes que las producen. En el aislamiento se detectaron fenotípica y genéticamente la aerolisin/hemolisina, la enterotoxina citotónica labil (alt), las lipasas y las DNAsas. Mientras que los genes de la enterotoxina citotónica termoestable (ast) y el flagelo lateral (laf) no se detectaron. La cepa mostró susceptibilidad a cefalosporinas y quinolonas. En el presente artículo se documenta el potencial virulento de la cepa aislada y la posible importancia clínica de cepas de esta genoespecie. A pesar de la aparente baja frecuencia de aislamiento de cepas *A. hydrophila* del HG 3, éstas no deben subestimarse, ya que en general muestran el mismo potencial virulento y perfil de resistencia que las especies de *Aeromonas* que exhiben mayores frecuencias de aislamiento. El presente trabajo aporta información al conocimiento de estas cepas tan particulares.

Key words: *Aeromonas*, identification, virulence, diarrhea.

Palabras clave: *Aeromonas*, identificación, virulencia, diarrea.

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Sponsorships. M.G.A.A., GCE, and CHR received COFAA, EDI and SNI supports.

This work was supported by grants from SIP 553 and 727 IPN, Mexico.

Recibido: 11-05-2009

Aceptado: 05-10-2009

INTRODUCTION

The *Aeromonas* genus exhibits an important genomic diversity; in the last edition of Bergey's Manual of Systematics Bacteriology, 14 phenospecies of mesophilic motile aeromonads that correspond to at least 17 DNA hybridization groups or genomospecies were recognized. There is considerable evidence that some strains, particularly the HG1, 4, 8/10, 9, 12, and 14 are pathogens for humans and have been accurately grouped in three bio-complexes: *Aeromonas hydrophila* complex, *A. caviae* complex, and *Aeromonas veronii* complex. The *Aeromonas hydrophila* complex includes four DNA/DNA hybridization groups (HG) that represent four different species, *A. hydrophila* *sensu stricto* HG1, *A. bestiarum* HG2, *A. salmonicida* HG3, and *A. popoffii* (HG17).^{1,2} Members of the originally defined *A. salmonicida* species group are currently contained in four groups: i) Non-motile, psychrophilic typical strains represented by *A. salmonicida* subsp. *salmonicida*; ii) Non-motile, psychrophilic atypical strains, including *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *smithiae*;^{2,3} iii) Non-motile, mesophilic strains that belong phenotypically and genetically to the *A. salmonicida* species, but, according to their phenotypic properties, they constitute a new *A. salmonicida* subspecies; *A. salmonicida* subsp. *pectinolytica*;⁴ and iv) motile, mesophilic strains genetically related to the *A. salmonicida* species but with phenotypic traits similar to those of *A. hydrophila*. Based on historical findings, this group is unsuitably referred as *A. hydrophila*-like (HG3) but it awaits proper taxonomic allocation.^{5,6}

Previous studies described the isolation of HG3 strains (phenotype *A. hydrophila*) from the environment⁷⁻¹⁰ and from feces of patients with diarrhea at a low incidence.^{5,7-9,11-14} In contrast, *A. hydrophila* *sensu stricto* HG1 is found as one of three *Aeromonas* species predominantly recovered from clinical samples and it has been involved in a wide array of intestinal and systemic infections.^{12,15} In addition, *A. hydrophila* is a bacterium commonly found in freshwater streams and ponds, frequently causing internal and generalized fish infections, particularly in fish farming.

Earlier studies have reported the most common *Aeromonas* species isolated from diarrhea samples from the state of Hidalgo and Mexico City.¹⁶⁻¹⁸ Like in other countries, 89.5% of the *Aeromonas* strains genetically identified in Mexico corresponded to *A. caviae*, *A. hydrophila* *sensu stricto*, and *A. veronii*. The remainder 10.5% has been identified as *A. bestiarum*, *A. media*, and *A. trota*.¹⁸ The aim of this study was to

characterize an isolate of *A. hydrophila*-like HG3 strain isolated from loose diarrhea of a one-year-old infant from Acaxochitlán, state of Hidalgo, Mexico. The patient had four loose evacuations per day during 3 days before health improvement. To our knowledge, this report is the first for *A. hydrophila*-like HG3 isolated from diarrhea stools in Mexico.

METHODS

Bacterial isolation

The strain 242-Hgo isolated in a clinical laboratory from the state of Hidalgo, Mexico, was recovered in pure culture from stool specimen, identified and labelled as *Aeromonas* spp. The phenotypical genus identification was based on the following traits: oxidase positive, Gram-negative rods, resistance to 150 µg/mL vibriostatic agent (2, 4 diamino-6,7 diisopropylpteridine; Oxoid, United Kingdom), glucose fermentation on O/F medium, growth on 3.5 and 6.0 % NaCl, and acid production from inositol.

Phenotypic identification

i. Conventional test

The strain was biochemically identified to the species level using the tests previously described:⁵ indole, mobility, gas from glucose, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase by the Moeller's method, esculin hydrolysis, Voges-Proskauer, acid production from L-arabinose, lactose, sucrose, salicin, m-inositol, D-mannitol, and production of β-hemolysin test. The entire tests were assayed at 20, 30 and 37°C.

ii. API 20E system

The strain was identified by API 20E system (bioMérieux, Lyon, France). The strain was taken from the surface of tryptic soy slant agar (Difco Laboratories, Detroit, MI), streaked onto the surface of a 5.0% sheep blood agar plate (tryptic soy agar base, Difco Laboratories, Detroit, MI), and incubated overnight at 28°C and 37°C. Several isolated colonies from this second plate were then used to inoculate a 5-mL tube of 0.85% sterile saline to approximate the 0.5 McFarland tube (1.5×10^8 CFU/mL). Two API 20E strips were inoculated according to manufacturer's instructions (bioMérieux, France) and incubated at 28°C and 37°C. After 24 h of incubation, reagents

were added to the API 20E strips, and a seven-digit profile number was generated for test interpretation.

iii. Specific test

The acid production from D-rhamnose, D-sorbitol, lactose, and the N-acetyl-glucosamine utilization was tested employing conventional protocols. DL-lactate,¹⁹ urocanic acid,²⁰ elastase,²¹ and gluconate oxidation²² were assayed as previously described. The entire tests were assayed at 20, 30 and 37°C.

16S rRNA-RFLP genetic identification

RFLP patterns were used for the identification of the genetic species. Briefly, the PCR-amplified 16S rRNA gene (1502 bp) was digested using the methods previously described. The amplicon was digested with two endonucleases (*Alu*I and *Mbo*I, Invitrogen) simultaneously; the obtained profile was common for *A. bestiarum*, *A. salmonicida*, *A. popoffii*, and *A. encheleia*. Thus, to differentiate the strain, further digestion with *Nar*I (Invitrogen) was necessary. A pattern profile (1050 bp and 452 bp fragments) congruent with the *A. salmonicida*/*A. bestiarum* identification was observed. Finally, digestion with *Pst*I enabled the identification of the strain as *A. salmonicida* since the 16S rRNA gene was not digested.^{23,24}

Susceptibility testing

Antimicrobial susceptibility tests were performed by using an agar disk diffusion method advocated by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS). Antimicrobial disks tested were: amikacin (AN 30 µg), ampicillin (AM 10 µg), cefotaxime (CTX, 30 µg), cefuroxime (CXM, 30 µg), cephalothin (CF, 30 µg), chloramphenicol (C, 30 µg); ciprofloxacin (CIP, 5 µg), gentamicin (GM, 10 µg), kanamycin (K 30 µg), imipenem (IPM, 10 µg), nitrofurantoin (F/M, 300 µg), polymyxin (PB, 300 µg), piperacillin (PIP, 100 µg), rifampicin (RA, 5 µg), tetracycline (TE, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg) (Becton Dickinson, México). *A. hydrophila* ATCC 7966^T, *A. caviae* ATCC 15468^T, *A. veronii* bv. *sobria* ATCC 35624^T, and *E. coli* ATCC 25922 strains were used as quality-control strains.²⁵

Phenotypic determination of virulence factors

Protease activity was assayed by spreading *Aeromonas* strains on nutrient agar containing 10% skim

milk. After incubation for up to 24 h at 28 and 37°C, protease production was shown by the formation of a clear zone caused by casein degradation. Gelatinase production was assayed by a radial diffusion method using slants containing 3% gelatine in 1% agarose gel. Ten microliters of each suspension was placed on the medium; the plates were then incubated 24 h at 28 and 37°C and cooled for 5 h at 4°C. Then, the plates were immersed in a saturated ammonium sulphate solution at 70°C to precipitate non-hydrolyzed gelatine. The appearance of a transparent zone around the colonies was considered positive for gelatinase activity. Hemolysin production was assayed by streaking the strain onto the surface of a 5.0% sheep blood agar plate (blood agar base) and incubating at 28° and 37°C for 24 h. The appearance of a clear colorless zone surrounding the colonies indicated β-hemolytic activity. Lipase activity was determined by two different methods: (i) 10 µL of an overnight bacterial suspension was placed on agarose dissolved in phosphate buffer saline containing 1% L-α-lecithin (Sigma, Barcelona, Spain) and incubated at 28 and 37°C during 96 h.²⁶ The presence of a transparent zone around the colonies indicated lipase activity; (ii) 5 µL of the overnight bacterial suspension was streaked onto resarurine-butter agar, the plates were incubated at 28 and 37°C for 24 h; the presence of pink colonies indicated lipase activity.¹⁸

PCR for virulence genes detection

PCR amplifications of *aer/hem*, *gcat*, *lip*, and *dnase* genes were performed with 2 µg of DNA using conditions²³ and primers previously described.²⁷ The *alt*, *ast*, and *lafA* genes were amplified as previously reported.²⁸ Negative controls, to which none DNA template was added, were also included in every set of reactions. *A. hydrophila* CECT 839^T and *A. caviae* CECT 838^T were used as positive controls. Identity of amplicons was confirmed by sequencing with an ABI-PRISM™ 310 following the standard methodology recommended by the manufacturer (Applied Biosystems).

Sequencing and phylogenetic analysis

The PCR product of the previously amplified 16S rRNA gene was sequenced with an ABI-PRISM™ 310 following the standard methodology recommended by the manufacturer (Applied Biosystems). The analysis of the obtained sequence was performed with SeaView²⁹ and BioEdit vers. 5.0.9 software. For phylogenogram purposes, the 16S rRNA sequence of the 242-

Hgo strain and related sequences obtained from the NCBI were analyzed: AB027544, AY910844, AM296506, X74680, AY987755, AY987756, AB027005, AJ009859, NC_009348 (*rrsA* to *H*), AY987757, X74681, AF134065, AJ223181, X60404, X60407, X60408, AY910844, AM296505, AJ223180, AJ224308, AJ508766, AF099021, AY686711, AY987759, AJ508765, AY987725, EU082830, X60409, and DQ092333. Multiple alignment analyses were performed with CLUSTAL X³⁰ to align the sequences. The weighting transversion/transition, using the Kimura 2 parameter model,³¹ and the number of base substitutions between each pair of sequences were estimated using the program MEGA vers. 4.³² The phylogenetic trees were constructed using the Neighbour-Joining method and the Kimura 2 parameter model of distance analysis, and 1000 bootstrap replications were assessed to support internal branches. Thus, the 16S rRNA gene amplified from *A. salmonicida* strain was sequenced and compared with those sequences obtained from the database. The sequence data reported in this work have been deposited on the EMBL database, under the accession number: AM931169.

RESULTS

Initially, the 242-Hgo strain was phenotypically identified by both the API system and phenotypic conventional tests. The results showed that they belonged to *A. hydrophila*. The genetic identification, based on 16S rRNA-RFLP profile, revealed that the strain was *A. salmonicida*. Since this is not a commonly isolated species from diarrhea, the strain was subjected to re-identification with the same three methods. The result was identical on the second time. The strain was motile and mesophilic, an *A. salmonicida* specific biochemical test was performed for the differentiation from *Aeromonas hydrophila* complex (hybridization groups, mainly 1, 2, and 3). DL-lactate and rhamnose were not assimilated, N-acetyl-D-glucosamine and urocanid acid were used as sole carbon and energy sources. Positive gluconate oxidation and acid production from sorbitol and lactose were detected. Elastase production was confirmed and a faint yellow pigment at both 28 and 37°C was observed after 3 days. Table 1 shows the biochemical responses obtained with the 242 Hgo strain, results of the type and reference strains and two atypical *A. salmonicida* strains were

Table I. Tests for the phenotypic differentiation of *A. salmonicida* from other species of the *A. hydrophila* complex.

Species	HG	Urocanic acid	Gluconate oxidation	DL-lactate	Sucrose	Rhamnose	Sorbitol	Lactose	Esculin hydrolysis	Motility	Salicin	N-acetyl-D-glucosamine	Citrate	Lysine decarboxylase	Elastase	Indole	Growth at 20-22°C	Growth at 30°C	Growth at 37°C
<i>A. hydrophila</i>																			
ATCC 7966 ^T	1	-	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	+	+
<i>A. bestiarum</i>	2	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+
CECT 4227 ^T	2	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CECT 894 ^T	3	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> CECT 895 [*]	3	-	-	-	+	+	-	-	+	-	-	-	-	-	+	-	+	+	+
<i>A. salmonicida</i> subsp. <i>masoucida</i> CECT 896 [*]	3	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+
<i>A. salmonicida</i> subsp. <i>smithia</i> NCIMB 13210 [*]	3	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+
<i>A. salmonicida</i> subsp. <i>pectinolytica</i> 34 MEL [*]	3	+	-	-	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+
<i>A. hydrophila</i> LMG 13451 [*]	3	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
<i>A. hydrophila</i> 242 Hgo	3	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Atypical <i>A. salmonicida</i> AS4	3	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	+	+	+
Atypical <i>A. salmonicida</i> AS222	3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-

With exception of the 242 Hgo strain, data were taken from Martínez-Murcia *et al.*, 2005. ^TType strain, ^{*}Reference strain.

added to point out the differences among the species that conform the *A. hyrophila* complex. The entire test confirmed the identity of the strain as *A. hyrophila*-like HG3. The characterization was confirmed by sequencing the 16S rRNA gene (Figure 1). The unrooted phylogenetic tree was constructed with 1461 nucleotides, it shows a clear clustering of the 242 Hgo strain inside the *A. salmonicida* species.

Several virulence potential attributes were found in the strain. Proteases (caseinase and gelatinase test), lipases (1% L- α -lecithin and resarsurine-butter agar), and hemolysins production were detected by *in vitro* assays. The strain harbored *aer/hem*, *alt*, *gcat*, *lip*, and *dnase* genes; whereas *laf* and *ast* genes were not detected. The strain resulted susceptible to most antimicrobials tested, including first-generation qui-

nolones and second- and third-generation cephalosporins, but resistant to rifampicin, cephalothin, ampicillin, and piperacillin.

DISCUSSION

Increasing numbers of *Aeromonas* clinical isolates are being reported, mainly from acute diarrhea cases. The current increase in frequency is presumably due to a greater awareness, better isolation methods, or because of an actual increase in the prevalence of *Aeromonas*. The improved isolation and identification tests have been decisive and have provided better epidemiological information.

Currently, there is substantial information about the main species infecting humans. However, there is

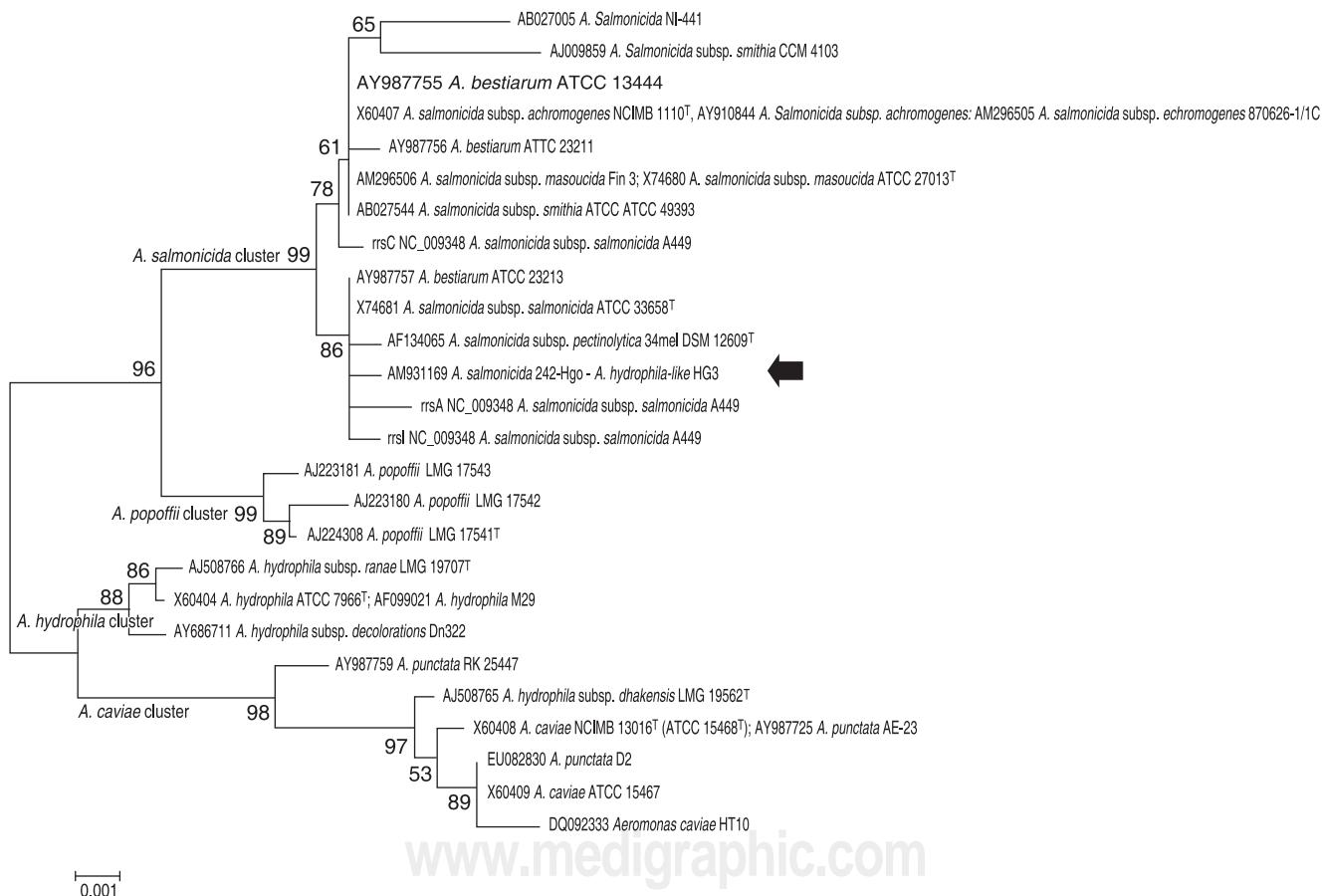


Figure 1. Phylogenetic relationships between the 16S rRNA gene sequence of the 242-Hgo strain and those members of the *Aeromonas* genus. *A. caviae* was used as an *A. hyrophila* complex outgroup. Bootstrap values above 50% are indicated in the main nodes in a bootstrap analysis of 1000 replicates. The scale bar represents the expected number of substitutions over all sites analyzed. The construction was performed based on a 1461 bp comparison using neighbor-joining method and the Mega program version 4. The arrow points out the 242-Hgo strain, > 99% of similarity of sequence with *A. salmonicida* species was obtained.

not enough information concerning those species isolated at lower frequencies. At present, only certain intraspecific subsets of *Aeromonas hydrophila*, *A. caviae*, and *A. veronii*, supplied with certain genes for enterotoxicity, are significantly associated with diarrhea, whereas *A. schubertii*, *A. bestiarum*, *A. media*, *A. jandaei*, *A. trota*, and *A. salmonicida*, including motile mesophilic strains and *A. hydrophila* HG3 strains from diarrheic samples, have been poorly characterized.

Human gastrointestinal infections due to *A. salmonicida* (motile mesophilic strains) have been reported at a lower frequency^{6,13,33} and few articles have described their virulence potential. Certainly, this low frequency could be a misidentification issue, perhaps they have not been properly identified because only few laboratories use phylogenetic approaches in their analyses and the phenotypic test does not allow for their correct identification. Nevertheless, in Mexico, the *Aeromonas* incidence reports have been around 2.1%. *A. hydrophila* species *sensu stricto*, *A. caviae*, and *A. veronii* are the most frequently isolated species and usually represent more than 89.5% of the *Aeromonas* clinical isolates genetically identified.¹⁷ In contrast, the 242 Hgo strain is the first and single *A. hydrophila*-like HG3 isolate, its low frequency of isolation in our country is still under discussion. In *A. salmonicida* strains, only cytotoxin production^{9,13,34} has been assessed, but the presence of other recognized potential virulence factors should be considered relevant information for the understanding of both their pathogenesis and epidemiology.¹⁶ In the present study, we explored the presence of genes encoding three of the most prominent *Aeromonas* toxins, and of genes encoding for lateral flagella and other putative virulence factors, including lipases, glycerophospholipid-cholesterol acyltransferase, and DNase. The virulence attributes and the antimicrobial resistances observed in the 242-Hgo strain were similar to those detected in *A. salmonicida* strains isolated from frozen fish intended for human consumption in Mexico.¹⁸ In contrast to *Aeromonas* of major clinical relevance, which exhibited multi-drug resistance,^{11,35,36} no relevant antimicrobial resistances were found in the 242-Hgo strain with the agar disk diffusion method. In agreement with previous Mexican studies, quinolones and second- and third-generation cephalosporins were the drugs with the best antimicrobial effect on *Aeromonas* species.^{11,18} However, the detection of reduced susceptibility in some geographic areas could indicate that aeromonads are becoming resistant to these drugs.^{35,36} For this reason, the rational utilization of

antimicrobials is important to avoid the appearance of high levels of resistance in the near future.

Sequencing of the 16S rRNA gene is considered a robust taxonomic tool. In *Aeromonas*, this gene provides also signature regions for the delineation and identification of most species, but for some species this does not guarantee the differentiation, since the sequence was found to be extremely conserved; for example, *A. salmonicida* and *A. bestiarum* type strains differ by only two nucleotides, at positions 1011 and 1018. To avoid possible doubts about the identity of the 242 Hgo strain, the DNA 16S rRNA sequence similarity and phylogenetic analyses were performed. We compared the 242-Hgo strain with the *A. salmonicida* subspecies and more than 99% of similarity was observed. In addition, the 16S rRNA RFLP pattern and the T/A nucleotide combination at positions 1011 and 1018 were also compatible with *A. salmonicida* and not with *A. bestiarum* or *A. salmonicida*/*A. bestiarum* profiles. Besides, we found a minor but consistent phenotypic variation (five tests). All the evidence allows us to confirm that the 242-Hgo strain belongs to *A. hydrophila*-like HG3.^{6,37}

Surely, in the next years *A. hydrophila*-like HG3 will be correctly located within the *Aeromonas* taxonomy. Therefore, gathering information for the complete characterization of this kind of strains will be very valuable in subsequent studies when the proper taxonomic designations begin to be analyzed and used appropriately.

ACKNOWLEDGEMENTS

M.G.A.A., GCE, and CHR received COFAA, EDI and SNI supports. This work was supported by grants from SIP 553 and 727 IPN, Mexico. We thank Colección Española de Cultivos Tipo and Dra. Miroslava Sánchez from Laboratorio de Salud Pública of Hidalgo, Mexico, for providing the isolates.

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