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Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the *fstA* (ferric siderophore receptor) gene

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ABSTRACT

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29 The aims of the study were to evaluate a new PCR protocol designed to detect
30 *Aeromonas salmonicida* in fish tissues and to develop a non destructive method for the
31 diagnosis of furunculosis. A set of primers (Fer3, Fer4), flanking a fragment of the *fstA*
32 gene (coding for the ferric-siderophore receptor) was designed, showing to be sensitive
33 and specific. When compared to PCR methods previously reported, the new protocol
34 recognised all the 69 *A. salmonicida* strains evaluated, with no cross-reactions with the
35 other bacterial species analysed. Sensitivity assays were performed in fish tissues
36 seeded with serial dilutions of pure cultures of *A. salmonicida* and mixed cultures of this
37 bacterium with *Vibrio anguillarum* and *A. hydrophila*. Detection limits obtained were of
38 60 and 450 bacterial cells 100mg⁻¹ of tissue respectively. Mucus and blood were
39 evaluated in order to develop a non-destructive tool to detect the pathogen. The
40 detection limits in seeded mucus and blood samples were 2.5 x 10² and 1 x 10⁵ bacterial
41 cells mL⁻¹, respectively. When the method was used to detect *A. salmonicida* in
42 asymptomatic wild salmon, 4 samples of mucus and 6 of blood were positive,
43 corresponding to 6 out of the 31 fish examined, whereas only one of the samples
44 resulted positive by culture methods. It is concluded that the PCR protocol evaluated is
45 fast, specific and sensitive to detect *A. salmonicida* in infected and asymptomatic fish,
46 and will be helpful for the control of the disease through the prompt detection of carriers
47 within fish populations.

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53 **Key Words:** *Aeromonas salmonicida*, PCR, *fstA* gene, Non-lethal diagnosis.

54 **1. INTRODUCTION**

55 *Aeromonas salmonicida* is a well known fish pathogen causing heavy losses in
56 both marine and freshwater aquaculture. The pathogen presents a wide geographical
57 distribution including numerous European countries, USA, Canada, Australia, Japan
58 and the mainland of Asia (Austin and Austin, 2007). Once it was recognised only to
59 infect salmonids, but it has also been isolated from non salmonids marine and
60 freshwater fish including turbot (*Scophthalmus maximus* L.), gilthead seabream (*Sparus*
61 *aurata* L.), lamprey (*Petromyzon marinus* L.) and Atlantic cod (*Gadus morhua* L.)
62 (Austin and Austin, 2007). *A. salmonicida* is commonly detected in mature wild salmon
63 entering the rivers in the Atlantic US coast (Cipriano *et al.*, 1996) as well as in the
64 Atlantic Coast of Spain (Ortega *et al.*, 2005), which could be one of the reasons for the
65 decline of wild populations in this area where *A. salmonicida* is also considered to be a
66 limiting factor in the turbot production.

67 In the past decade there has been much interest in the development of specific
68 PCR protocols, many of them based on the amplification of 16S rRNA genes, for
69 detecting a variety of Gram negative and Gram positive bacterial fish pathogens in fish
70 samples and complex substrates (Brown *et al.*, 1994; Cunningham, 2002; Romalde and
71 Toranzo, 2002), including *A. salmonicida* (Del Cerro *et al.*, 2002; Gustafson *et al.*,
72 1992; Hiney and Smith, 1998; Høie *et al.*, 1999; Miyata *et al.*, 1996; O'Brien *et al.*,
73 1994). False negative results have been described for detection of *A. salmonicida* using
74 some of these methods (Byers *et al.*, 2002; Ramos, 2006), being recognized that the
75 primer set designed by Gustafson *et al.* (1992) give the best results. On the other hand,
76 most procedures of diagnosis require the sacrifice of the animal and, to our knowledge,
77 no studies have been performed to evaluate the suitability of PCR for the detection of *A.*
78 *salmonicida* in fish samples without causing death to the animal.

79 Therefore the objective of this study was to develop an improved PCR method
80 based on new primers for the rapid and specific diagnosis of furunculosis caused by *A.*
81 *salmonicida*. The genes *gyrA* and *fstA* were selected as markers because of their
82 importance for the viability of the pathogen. The bacterial enzyme DNA gyrase
83 (topoisomerase II) catalyzes the introduction of negative supercoils into covalent closed
84 circular DNA and is thereby essential for a viable bacteria (Reece and Maxwell, 1991).
85 The gene *fstA* is implicated in the functioning of the ferric siderophore receptors which
86 are in charge of the uptake of iron, an essential mechanism for the survival of the

87 bacteria within the host. The applicability of the new protocol in different fish tissues
88 was also studied in order to develop a non-destructive diagnosis approach.

89

90 **2. MATERIALS AND METHODS**

91 ***2.1. Bacterial strains and growth conditions.***

92 A total of 69 *A. salmonicida* strains, including 66 *A. salmonicida* subsp.
93 *salmonicida* isolates, 1 *A. salmonicida* subsp. *masoucida*, 2 *A. salmonicida* subsp.
94 *achromogenes*, were used in this study. The sources of isolation and geographical origin
95 of these strains are listed in Table 1. Representatives of other *Aeromonas* species, as
96 well as isolates of different bacterial fish pathogens were used to develop and optimize
97 the PCR assay (Table 2).

98 Strains were routinely grown in Tryptic Soy Agar (Pronadisa, Madrid, Spain)
99 supplemented with NaCl to a final concentration of 1% (TSA-1), except for
100 *Pseudomonas anguilliseptica* and *Lactococcus garvieae* that were grown on Columbia
101 sheep blood agar (CBA) (Oxoid Ltd., Madrid, Spain) and *Tenacibaculum maritimum*
102 that was grown on *Flexibacter maritimus* Medium (FMM) agar (Pazos *et al.*, 1993).
103 Strains were incubated at 22-25 °C for 48 h. Stock cultures were maintained frozen at -
104 80 °C in Microbank™ cryovials (Pro-Lab Diagnostics, Ontario, Canada).

105

106 ***2.2. DNA extraction and design of the primer set.***

107 DNA was extracted from pure bacterial cultures employing the Insta-Gene
108 matrix (Bio-Rad, Madrid, Spain) as previously described by Romalde *et al.* (1999).
109 DNA concentration, espectophotometrically measured, ranged from 3.5 to 4 µg/µl.
110 Purified DNA was stored at -20 °C until use. One µL of the DNA suspension was
111 routinely used for each PCR when working with pure cultures.

112 The sequence of the *fstA* gene (accession N° X87995) and of the *gyrA* gene
113 (accession N° L47978) of *A. salmonicida* were retrieved from the Genbank database and
114 compared with the closest relatives employing the MegaBlast software. On the basis of
115 the alignment, regions were chosen to be the most variable in comparison with *A.*
116 *hydrophila*, the only species from the genus *Aeromonas* with available sequence data
117 for these genes. For a PCR assay three pairs of primers were designed for the gene *fstA*,
118 and one pair for the *gyrA* gene (Table 3). The specific primers designed were
119 synthesized by Sigma-Genosys (London, UK). Primers were evaluated and one pair
120 selected on the basis of their specificity and sensitivity.

121 **2.3. Optimization of the PCR amplification.**

122 The PCR protocol was optimized by amplification reactions in a T-gradient
123 thermal cycler (Biometra, Goettingen, Germany) using the Ready-To-Go PCR beads
124 (Pharmacia Biotech, Barcelona, Spain) which included all the reagents needed for the
125 PCR reactions (nucleotides, buffer and *Taq* DNA polymerase). Reaction mixtures had 1
126 μL of each primer (2 μM), 1 μL of the DNA template (unless otherwise cited) and 22
127 μL of sterile distilled water (final volume of 25 μL). One *A. salmonicida* strain (ATCC
128 14174) and two *A. hydrophila* strains (020116-03, 010530-01) were used for the
129 optimization reactions.

130 PCR conditions consisted of an initial denaturation step at 92 °C for 3 min
131 followed by 30 cycles of amplification in which denaturation and elongation
132 temperatures were 92 °C for 1 min and 72 °C for 1 min respectively, the annealing
133 temperatures ranged from 55 °C to 65 °C for 1 min. A final elongation step of 72 °C for
134 5 min was included in the program.

135 Amplification products were analyzed on 1% (wt/vol) agarose gels with TAE
136 (0.04 M Tris, 0.0001M EDTA, pH 8.0) electrophoresis buffer and were visualized with
137 a UV transilluminator after staining with ethidium bromide (2 $\mu\text{g mL}^{-1}$). A 50- 2000 bp
138 ladder (Sigma Chemical Co., St. Louis, MO, USA) was used as a molecular mass
139 marker. Bands at expected sizes for each primer pair (Table 1) were considered a positive
140 result in each experiment.

141

142 **2.4. Determination of PCR specificity and sensitivity.**

143 The specificity of the primers was evaluated using the program with the
144 optimized PCR conditions. with all the *A. salmonicida* strains and the representatives of
145 the other bacterial species (Tables 1 and 2).

146 The DNAs from all *A. salmonicida* strains were amplified in parallel using the
147 primers designed here and the primers AP-1/2 designed by Gustafson *et al.* (1992),
148 targeting the *vapA* gene, which encodes a unique subunit protein (the A-protein) of the
149 A-layer of *A. salmonicida* (Table 1).

150 The specificity of the primer set Fer-3/Fer-4 was also assessed by sequencing of
151 the amplicons obtained. Thus, amplicons from different PCR reactions and DNA
152 extractions were randomly selected to be sequenced. Amplicons were purified using the
153 QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the

154 manufacturer's instructions, and subjected to sequencing reactions in a T-gradient
155 thermal cycler (Biometra) using the GenomeLab™ Dye terminator cycle sequencing
156 (DTCS) quick start kit (Beckmen Coulter, Fullerton, CA, USA). Sequencing products
157 were analysed using a Automatic DNA Sequencer CEQ8000 (Beckman Coulter).
158 Comparative sequence analyses were conducted using FASTA3 program (Pearson and
159 Lipman, 1988).

160 The detection limit of the primer sets designed were evaluated in sensitivity
161 assays using pure or mixed cultures as previously described (Avendaño-Herrera *et al.*,
162 2004). Separate bacterial suspensions of two *A. salmonicida* strains (ACR 117.1 and
163 ATCC14174) were prepared alone or mixed at equal proportion (1:1:1) with
164 suspensions of *Vibrio anguillarum* (R72) and *A. hydrophila* (RPM 747.1), and serially
165 diluted in 0.85% sterile saline solution from 10^8 to 10^0 cells mL^{-1} . Colony forming units
166 per milliliter (CFU mL^{-1}) were estimated in all cases by plating onto TSA-1 medium. All
167 the dilutions of pure or mixed cultures were centrifuged at $12000 \times g$ for 2 min and
168 DNA was extracted by the Insta-Gene matrix. Conditions for PCR amplification and
169 electrophoresis were the same as described above.

170

171 **2.5. Determination of PCR sensitivity in fish tissues.**

172 PCR sensitivity was also determined employing DNA extracted from *in vitro*
173 seeded kidney and skin tissues as previously described (Avendaño-Herrera *et al.*, 2004).
174 Briefly, tissues were collected from healthy turbot in aseptic conditions and divided into
175 200 mg portions. Each sample was seeded with 100 μL of different dilutions (from 10^7
176 to 10^2 cells mL^{-1}) of pure or mixed cultures prepared as described above in saline
177 solution (0.85%), and homogenized for 60 s. After incubation for 1 hour, DNA
178 extraction was performed with the kit EASY-DNA (Invitrogen, Carlsbad, CA, USA)
179 following the manufacturer's instructions. Unseeded tissues were used as negative
180 controls and were processed for the DNA purification protocol in the same way. For the
181 PCR, 5 μL of the purified DNA were added as the template.

182

183 **2.6. Non-destructive PCR protocols.**

184 Mucus (50 μL) and blood (100 μL) samples aseptically collected from the fish
185 surface or by venepuncture from anaesthetised animals (Avendaño-Herrera *et al.*, 2004)
186 were mixed with equal volumes of different dilutions (from 10^8 to 10^1 cells mL^{-1}) of an
187 *A. salmonicida* (ATCC 14174) culture suspension in saline solution (0.85%). DNA

188 purification was performed with InstaGene Matrix. Unseeded blood and mucus samples
189 were used as negative controls and were processed in the same manner. For the PCR, 5
190 μL of the purified DNA were added as the template. Conditions for PCR amplification
191 and electrophoresis were the same as described above.

192 Samples of mucus and blood were taken from 31 wild salmon captured in
193 different rivers to serve as broodstock and were immediately analyzed for the presence
194 of the pathogen by the PCR protocol described. In all cases, classical culture - based
195 diagnosis including isolation onto TSA-1 agar and biochemical identification by the
196 miniaturized API 20E system was performed in parallel.

197

198 3. RESULTS

199 Of the four sets of primers that were evaluated, Fer-1/Fer-2 did not amplify
200 DNA from *A. salmonicida* strains, and Fer-5/Fer-6 amplified DNA from strains of *A.*
201 *hydrophila* with a same size band as DNA from *A. salmonicida* (data not shown). These
202 two pair of primers were discarded. The pair of primers Fer-3/Fer-4, and A_{sg}-1/A_{sg}-2
203 did amplify correctly and specifically. Primers Fer-3 and Fer-4 were found to have a
204 better sensitivity limit, so they were used to perform all the amplification reactions in
205 this study.

206 The conditions of the PCR protocol employing the primer set Fer-3/Fer-4 were
207 optimized by using *A. salmonicida*, ATCC14174 and two *A. hydrophila*, 020116-03,
208 010530-01. A unique PCR band of the expected size 422 bp appeared at all the
209 annealing temperatures (55 to 65 °C) tested with the *A. salmonicida* strain, while no
210 bands or high molecular size products were observed for the *A. hydrophila* isolates (data
211 not shown). On the basis of these results, 60 °C was the annealing temperature chosen
212 for the subsequent PCR reactions. The following conditions were fixed for the designed
213 PCR protocol: an initial denaturation step for 3 min at 92 °C, 30 amplification cycles,
214 with denaturation for 1 min at 92 °C, annealing for 1 min at 60 °C and elongation for 1
215 min at 72 °C, followed by a final extension step of 5 min at 72 °C .

216 The specificity of the primers Fer-3 and Fer-4 was assayed by using 69 strains of
217 *A. salmonicida* and 38 of other bacterial species. All the *A. salmonicida* strains yielded
218 the expected amplification product of 422 bp under the conditions chosen for the PCR
219 programme (Table 2, Fig. 1 and 2a). Non-specific amplifications were obtained for
220 some *A. hydrophila* strains whereas no amplification products were observed for any of
221 the other fish pathogens analyzed (Table 2, Fig. 1). Sequencing of 15 amplicons

222 randomly selected confirmed the specificity of the PCR, in all cases a similarity higher
223 than 95.5% was obtained with the *fstA* gene sequence (GenBank accession N° X87995).
224 The 69 *A. salmonicida* strains were also tested with the primer set AP-1/AP-2.
225 Amplification products were obtained for 64 out of the 69 strains analyzed. The five *A.*
226 *salmonicida* strains not recognized, included two isolate from lamprey, one from
227 salmon, one from turbot, and a reference strain (Fig. 2b). Neither pair of primers was
228 able to discriminate among subspecies of *A. salmonicida* subsp. *salmonicida*, *A.*
229 *salmonicida* subsp. *masoucida* and *A. salmonicida* subsp. *achromogenes*.

230 A series of experiments were performed to determine the sensitivity of the
231 primers Fer-3 and Fer-4. The detection limit in pure cultures of *A. salmonicida* was
232 between 20 and 200 cells mL⁻¹ (Fig. 3a). In mixed cultures the limit found was between
233 60 and 600 cells mL⁻¹ (data not shown). The PCR protocol was performed to DNA
234 extracted from fish tissues (kidney and skin) seeded with different concentrations of *A.*
235 *salmonicida*. The detection limit achieved was of 0.6-6 x10² bacterial cells per 100 mg
236 of tissue when seeding a pure bacterial suspension (Fig. 3b), and of 4.5-10 x 10² per
237 100 mg of tissue when a mixed bacterial culture was inoculated (Fig. 3c).

238 DNA extracted from mucus and blood samples seeded with different
239 concentrations of the bacteria were also submitted to the PCR assay. The lowest
240 detection limit obtained for mucus samples was a value of 2.5 x 10² bacterial cells mL⁻¹
241 of mucus (Fig. 4a), and for blood samples the lowest value detected was 1 x 10⁵ bacteria
242 mL⁻¹ of blood. It was observed that as the bacterial concentration decreased in the
243 blood, another amplicon of higher molecular weight appeared, inhibiting the expected
244 PCR product band (Fig.4b).

245 *A. salmonicida* was detected from mucus and blood of wild salmon without
246 signs of furunculosis using the developed PCR protocol for (data not shown).
247 Amplification of the specific PCR product was obtained in 6 (4 samples of mucus and
248 in 6 samples of blood) out of the 31 wild salmon examined. As in the case of pure
249 cultures, sequencing of these amplicons rendered similarities higher than 95.5% with
250 the original *fstA* gene sequence. Mucus and blood of these 6 carrier fish were assayed
251 on bacteriological media and it was only possible to isolate the pathogen from one
252 blood sample.

253

254 4. DISCUSSION

255 The culture-based diagnosis and biochemical identification of *A. salmonicida* is
256 tedious and time consuming. The difficulty of isolating the pathogen in the presence of
257 faster growing bacteria or when the fish are asymptomatic carriers are some of the
258 disadvantages of the classical bacteriological procedures. PCR techniques have shown
259 excellent advantages over these limitations. In fact, PCR detection protocols have been
260 developed as tools for diagnosis of many important fish diseases (Osorio and Toranzo,
261 2002; Romalde and Toranzo, 2002). Such approaches have been helpful in detection of
262 pathogens for both infected and asymptomatic carrier fish, and can constitute an useful
263 tool for the aquaculture of enhancement, such as the selection of pathogen-free
264 broodstocks.

265 A PCR procedure for detecting *A. salmonicida* was developed using the primers
266 Fer-3 and Fer-4. The *fstA* gene was selected as target, because of the better limit of
267 sensitivity shown by the primer set Fer-3/Fer-4 over the other primer pairs. The new
268 primers were able of specifically detecting *A. salmonicida* down to species level,
269 although distinguishing among subspecies was not possible. No cross-reactions with
270 any of the non-target organisms were observed. Moreover, the new designed primers
271 detected all the *A. salmonicida* isolates included in the study, regardless their origin or
272 year of isolation. It is important to note that there are no evidences of epidemiological
273 linkage among the strains, since they proceed from diverse farms and wild fish,
274 different years of isolations and from a variety of geographical areas. These findings
275 suggest the high specificity of the primers to detect *A. salmonicida*, which was
276 supported by the sequencing results obtained. The sensitivity experiments with pure
277 cultures demonstrated that the procedure possesses similar detection limits than a variety
278 of PCR methods designed for other bacterial fish pathogens (Hiney and Smith, 1998;
279 Osorio and Toranzo, 2002; Romalde and Toranzo, 2002).

280 In previous work, it has been described that primers AP-1/AP-2 (Gustafson *et*
281 *al.*, 1992) offered a good coverage in terms of identifying the *A. salmonicida*, although
282 these primers were also unable to distinguish between subspecies of this pathogen. In
283 terms of sensitivity, both sets of primers, AP1/AP2 and Fer-3/Fer-4, showed a similar
284 detection limit when applied to DNA extracted from pure cultures. However, in the
285 present study 6% of the *A. salmonicida* isolates were falsely recorded as negative,
286 which is in agreement with previous results obtained by Byers *et al.* (2002) and Ramos
287 (2006). The importance of the primer set has been highlighted in comparative studies
288 (Byers *et al.*, 2002), not in specificity but also in sensitivity. In this sense, the method

289 developed here to detect *A. salmonicida* in fish tissue yielded better sensitivity than
290 other described previously, being in the same range than those described using other
291 approaches, such as pre-enrichment associated with PCR or nested-PCR for *A.*
292 *salmonicida* (Taylor and Winton, 2002) and other pathogens like *Salmonella* spp. and
293 *Tenacibaculum maritimum* and (Avendaño-Herrera *et al.*, 2004; Bej *et al.*, 1994).

294 In addition, the detection limit obtained in mucus indicate its usefulness as a
295 valuable tool for a non-destructive diagnostic analysis. The results obtained in wild
296 salmon supported this finding. The detection of the pathogen in blood is also desirable,
297 especially in the case of asymptomatic carriers, since it can help to prevent outbreaks of
298 the disease. In this study, we were able to detect the bacteria from *in vitro* seeded blood
299 only at a very high concentration, probably due to interferences of the heparin or an
300 unknown blood component which competed with the bacterial DNA in the PCR
301 amplification. However, the method was able to detect *A. salmonicida* from blood of
302 asymptomatic wild salmon, presumably containing lower numbers of bacteria, which
303 could be explained by the immediate use of the samples. Similar results were obtained
304 for other pathogens such as *T. maritimum* (Avendaño Herrera, personal communication).
305 Further experiments to increase the sensitivity of the method in blood are needed, i.e.
306 the use of plasma instead whole blood could improve the sensitivity achieved in the *in*
307 *vitro* assays.

308 In summary, the method described here proved to be specific, sensitive and fast
309 in the detection of *A. salmonicida* in infected fish and asymptomatic carriers. Its
310 application as a non-destructive detection method using mucus as target tissue is
311 currently being used in the selection of broodstock of wild Atlantic salmon captured in
312 the rivers of Galicia to enhance these wild salmon populations.

313

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391 Table 1. *A. salmonicida* isolates^a included in this study.

Type of samples	Isolation source	Number of strains	No of strains identified by PCR ^b	
			Fer3-Fer4	AP1-AP2
Strains isolated from cultured fish	Turbot, Spain	30	30	30
	Turbot, Portugal	10	10	9
	Rainbow trout, Spain	5	5	5
	Atlantic salmon, Spain	2	2	1
	Atlantic salmon, Chile	1	1	1
Strains isolated from wild fish	Lamprey, Spain	4	4	2
	Atlantic salmon, Spain	8	8	8
	Sea trout, Spain	3	3	3
	Rainbow trout, Spain	2	2	2
Reference strains				
ATCC 14174	Atlantic salmon, USA	1	1	0
NCIMB 2261	Brook trout, Scotland	1	1	1
ATCC 27013 ^T	Pacific salmon, Japan	1	1	1
CECT 895 ^T	Brown trout, Scotland	1	1	1

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393 ^a Unless indicated, all isolates belong to the laboratory collection and were isolated between 1989 and 2005.
 394 ATCC, American Type Culture Collection, Manassas (VA), USA; NCIMB, National Collection of Industrial and
 395 Marine Bacteria, Aberdeen, Scotland; CECT, Spanish Collection of Type Cultures, Valencia, Spain.

396 ^b All strains have been described as *A. salmonicida* subsp. *salmonicida* with the exception of strain ATCC
 397 27013^T described as *A. salmonicida* subsp. *masoucida* and strains from Chilean Atlantic salmon and CECT 895^T
 398 described as *A. salmonicida* subsp. *achromogenes*

399 ^c Scientific names of fish species are: Turbot *Scophthalmus maximus*, Rainbow trout *Oncorhynchus mykiss*,
 400 Atlantic Salmon *Salmo salar*, Lamprey *Petromyzon marinus*, Sea trout *Salmo trutta fario*, Brook trout
 401 *Salvelinus fontinalis*, Pacific salmon *Oncorhynchus* sp., Brown trout, *Salmo trutta*.

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407 Table 2. Bacteria from other species included in this study as negative controls in the

408 PCR analysis.

Species	Strain	Donor ^a	PCR amplification Fer3/4
<i>Aeromonas bestiarum</i>	CECT 4227	CECT	NSA ^b
<i>Aeromonas hydrophila</i>	020116-03	Laboratory collection	NSA
<i>A. hydrophila</i>	055102-04	Laboratory collection	NSA
<i>A. hydrophila</i>	X.P. Alev.	Laboratory collection	–
<i>A. hydrophila</i>	TB 14.1	Laboratory collection	–
<i>A. hydrophila</i>	061002-01	Laboratory collection	NSA
<i>A. hydrophila</i>	PC 522.1	Laboratory collection	NSA
<i>A. hydrophila</i>	RPM 747.1	Laboratory collection	–
<i>A. hydrophila</i>	010530-01	Laboratory collection	NSA
<i>A. hydrophila</i>	B 32	Laboratory collection	NSA
<i>A. hydrophila</i>	TW 40.1	Laboratory collection	–
<i>A. hydrophila</i>	PC 521.2	Laboratory collection	NSA
<i>A. hydrophila</i>	053102-04	Laboratory collection	NSA
<i>A. sobria</i>	P33	Laboratory collection	–
<i>A. media</i>	ATCC 33907	ATCC	NSA
<i>A. caviae</i>	P322	Laboratory collection	–
<i>Vibrio harveyi</i>	RA 58.2	Laboratory collection	–
<i>V. anguillarum</i>	R 72	Laboratory collection	–
<i>V. anguillarum</i>	TM 20.1	Laboratory collection	–
<i>V. splendidus</i>	ATCC 33125	ATCC	–
<i>V. scophthalmi</i>	CECT 4638	CECT	–
<i>V. furnisii</i>	CECT 4203	CECT	–
<i>V. natriegens</i>	ATCC 14048	ATCC	–
<i>V. tubiashi</i>	ATCC 19106	ATCC	–
<i>V. nereis</i>	ATCC 25917	ATCC	–
<i>V. ordalii</i>	NCIMB 2107	NCIMB	–
<i>V. fischeri</i>	NCIMB 1274	NCIMB	–
<i>V. vulnificus</i>	ATCC 27562	ATCC	–
<i>Yersinia ruckeri</i>	TW 330	Laboratory collection	–
<i>Photobacterium damsela</i>	P12	Laboratory collection	–
<i>Tenacibaculum maritimus</i>	PC 560.1	Laboratory collection	–
<i>Marinomonas vaga</i>	NCIMB 1962	NCIMB	–
<i>Oceanomonas doudoroffi</i>	NCIMB 1965	NCIMB	–
<i>P. aeruginosa</i>	ATCC 27853	ATCC	–
<i>P. fluorescens</i>	ATCC 13525	ATCC	–
<i>P. anguilliseptica</i>	TW-P1	Laboratory collection	–
<i>Pseudomonas sp.</i>	RP 20.1	Laboratory collection	–
<i>Lactococcus garvieae</i>	TW-1	Laboratory collection	–

409 ^a CECT, Colección Española de Cultivos Tipo, Burjassot, Valencia, Spain; ATCC,
 410 American Type Culture Collection, Manassas (VA), USA; NCIMB, National Collection
 411 of Industrial and Marine Bacteria, Aberdeen, Scotland, UK.

412 ^b NSA, non-specific amplification.

413 **Table 3.-** Primer sets employed in this study.

Gen^a	Primer set	Sequence^b	Positions	Amplicon size	Reference
<i>fstA</i>	Fer-1 / Fer-2	5'CGCTCGCCCATCCCCCTCTG 3' (f) 5'GCCCCTTGCACCCCCACCATT 3' (r)	110 - 129 561 - 541	452	This work
<i>fstA</i>	Fer-3 / Fer-4	5'CGGTTTTGGCGCAGTGACG 3' (f) 5'AGGCGCTCGGGTTGGCTATCT 3' (r)	1683 -1701 2104 - 2084	422	This work
<i>fstA</i>	Fer-5 / Fer-6	5'AAGGGCCCGTCCGTGTTGCTCA3' (f) 5'ATACGGCTGCCCTGGTGCATT 3' (r)	886 - 907 1348 - 1327	463	This work
<i>gyrB</i>	Asg-1 / Asg-2	5'TGGCATGGAACATTCCCTCCT 3' (f) 5'GTCGCCTGCTTTTTCCAGCA 3' (r)	726 - 746 1485 - 1465	760	This work
<i>vapA</i>	AP-1 / AP-2	5'GGCTGATCTCTTCATCCTCACCC 3' (f) 5'CAGAGTGAAATCTACCAGCGGTGC3' (r)	1081 - 1108 1505 - 1482	421	Gustafson et al., 1992

414 ^a Target genes for the primer sets.415 ^b In parenthesis is indicated the sense of the primers: (f) forward and (r) reverse primer.

416 **Figure Legends**

417

418 **Fig. 1.** Specific PCR products with Fer-3 and Fer-4 for *A. salmonicida*, comparing with
419 other *Aeromonas* strains. Lanes: M, Amplisize Molecular Ruler (50-2000 bp ladder,
420 Sigma); 1 to 5 *A. salmonicida* strains PC 457.1, RI 80.1, RI 163, RSP 71.2, ACR 142.1,
421 6-10, *A. hydrophila* strains TB 14.1, PC 521.2, 055102-04, B32, 061002-01. Numbers
422 on the left indicate the position of molecular size marker in bp. Numbers on the right
423 indicate the size of the specific amplified product for *A. salmonicida*.

424

425 **Fig. 2.** Specific PCR products of 11 *A. salmonicida* strains. (A) Amplification with
426 primers Fer-3 and Fer-4. (B) Amplification with primers AP-1 and AP-2 (Gustafson et
427 al. 1992). Lanes: M, Amplisize Molecular Ruler (50-2000 bp ladder, Sigma); 1 to 11,
428 *A. salmonicida* strains, ASF1.1, 020601, RM 258.1, VT 42.1, SF 3.1/98, ATCC14174,
429 001218-01, 050202-02, ACR 117.1, L3R, SR 47.1. Numbers on the left indicate the
430 position of molecular size marker in bp. Numbers on the right indicate the size of the
431 specific amplified product for *A. salmonicida*.

432

433 **Fig. 3.** Sensitivity of the PCR protocol with Fer-3 and Fer-4 using purified DNA from
434 the isolate *A. salmonicida* ATCC 14174 (A), DNA extracted from fish tissues seeded
435 with different concentrations of *A. salmonicida* (ATCC 14174) in pure culture (B) and
436 with mixed cultures of *A. salmonicida* (ATCC 14174), *A. hydrophila* (RPM 747.1) and
437 *V. anguillarum* (R72)(C). Lanes in A: M, Amplisize Molecular Ruler (50-2000 bp
438 ladder, Sigma); 1 to 9, DNA extracted from serial decimal dilutions of the isolate ATCC
439 14174, ranging from 2×10^8 (Lane 1) to 2×10^0 (Lane 9) cells mL⁻¹. Lanes in B: M,
440 Amplisize Molecular Ruler (50-2000 bp ladder, Sigma); 1 to 6, amplified DNA
441 corresponding to 6×10^5 (Lane 1) to 6×10^0 (Lane 6) cells 100mg⁻¹, 7, negative control
442 (unseeded tissue). Lanes in C: M, Amplisize Molecular Ruler (50-2000 bp ladder,
443 Sigma); 1, negative control (unseeded tissues); 2 to 7, amplified DNA corresponding to
444 4.5×10^6 to 4.5×10^1 cells 100mg⁻¹. Numbers on the left indicate the position of
445 molecular size marker in bp. Numbers on the right indicate the size of the specific
446 amplified product for *A. salmonicida*.

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449 **Fig. 4.** Sensitivity of the PCR protocol in mucus (A) and blood (B) seeded with
450 different concentrations of *A. salmonicida* (ATCC 14174). Lanes in A: M, Amplisize
451 Molecular Ruler (50-2000 bp ladder, Sigma); 1, negative control (unseeded mucus); 2
452 to 9, amplified DNA extracted from seeded mucus samples ranging from 2.5×10^8 (Lane
453 2) to 2.5×10^1 (Lane 9) cells mL⁻¹. Lanes in B: M, Amplisize Molecular Ruler (50-2000
454 bp ladder, Sigma); 1, negative control (no DNA); 2 to 10, amplified DNA extracted from
455 seeded blood samples ranging from 1×10^8 (Lane 2) to 1×10^0 (Lane 10) cells mL⁻¹; 11,
456 negative control (unseeded blood). Numbers on the left indicate the position of molecular
457 size marker in bp. Numbers on the right indicate the size of the specific amplified
458 product for *A. salmonicida*.
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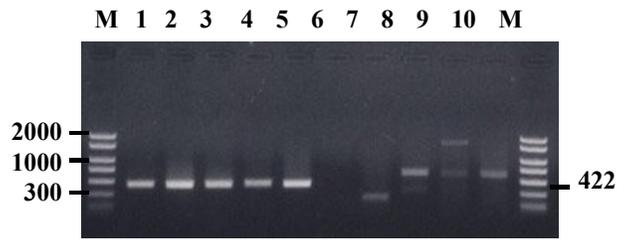


Fig. 1.- Beaz-Hidalgo et al.

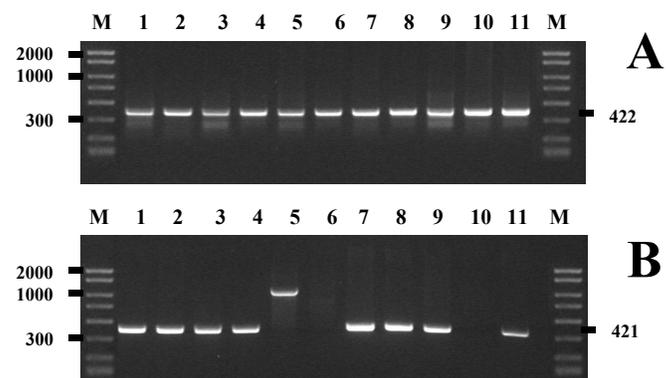


Fig. 2.- Beaz-Hidalgo et al.

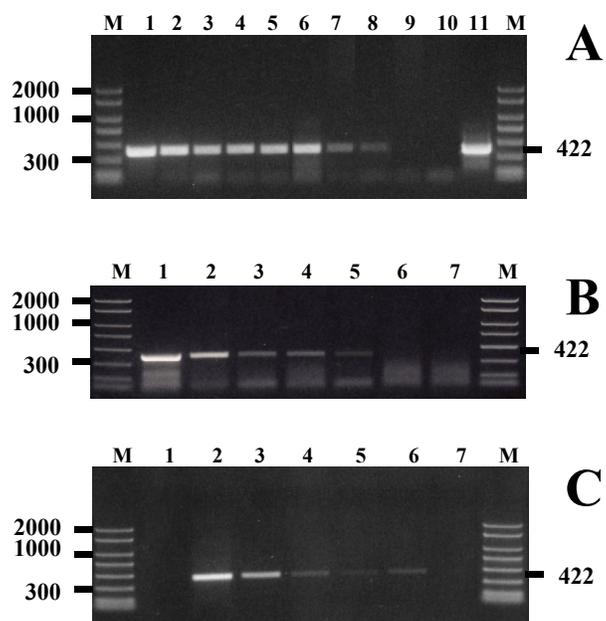


Fig. 3.- Beaz-Hidalgo et al.

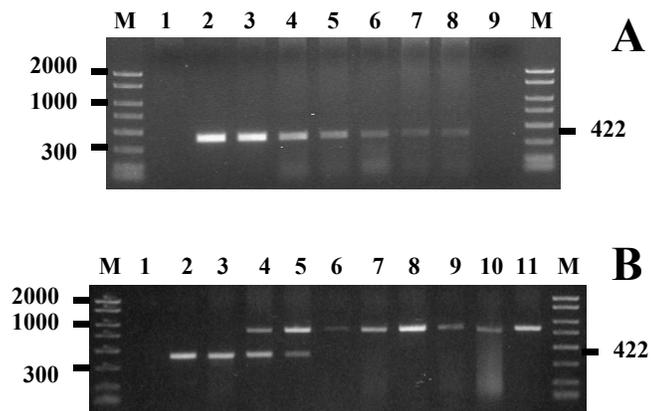


Fig. 4.- Beaz-Hidalgo et al.