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

The aims of the study were to evaluate a new PCR protocol designed to detect *Aeromonas salmonicida* in fish tissues and to develop a non destructive method for the diagnosis of furunculosis. A set of primers (Fer3, Fer4), flanking a fragment of the *fstA* gene (coding for the ferric-siderophore receptor) was designed, showing to be sensitive and specific. When compared to PCR methods previously reported, the new protocol recognised all the 69 *A. salmonicida* strains evaluated, with no cross-reactions with the other bacterial species analysed. Sensitivity assays were performed in fish tissues seeded with serial dilutions of pure cultures of *A. salmonicida* and mixed cultures of this bacterium with *Vibrio anguillarum* and *A. hydrophila*. Detection limits obtained were of 60 and 450 bacterial cells $100mg^{-1}$ of tissue respectively. Mucus and blood were evaluated in order to develop a non-destructive tool to detect the pathogen. The detection limits in seeded mucus and blood samples were 2.5×10^2 and 1×10^5 bacterial cells mL^{-1}, respectively. When the method was used to detect *A. salmonicida* in asymptomatic wild salmon, 4 samples of mucus and 6 of blood were positive, corresponding to 6 out of the 31 fish examined, whereas only one of the samples resulted positive by culture methods. It is concluded that the PCR protocol evaluated is fast, specific and sensitive to detect *A. salmonicida* in infected and asymptomatic fish, and will be helpful for the control of the disease through the prompt detection of carriers within fish populations.

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

1. INTRODUCTION

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

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

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

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

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions.

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

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

2.2. DNA extraction and design of the primer set.

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

The sequence of the *fstA* gene (accesion N^{o} X87995) and of the *gyrA* gene (accesion N^{o} L47978) of *A. salmonicida* were retreived from the Genbank database and compared with the closest relatives employing the MegaBlast software. On the basis of the alignment, regions were chosen to be the most variable in comparison with *A. hydrophila,* the only species from the genus *Aeromonas* with available sequence data for these genes. For a PCR assay three pairs of primers were designed for the gene *fstA*, and one pair for the *gyrA* gene (Table 3). The specific primers designed were synthesized by Sigma-Genosys (London, UK). Primers were evaluated and one pair 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 rections 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 poymerase). 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 ^{o}C for 3 min
131 followed by 30 cycles of amplification in which denaturation and elongation
132 temperatures were 92 ^{o}C for 1 min and 72 ^{o}C for 1 min respectively, the annealing
133 temperatures ranged from 55 ^{o}C to 65 ^{o}C for 1 min. A final elongation step of 72 ^{o}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 μg 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 cosidered 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 *vap*A 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

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

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

2.5. Determination of PCR sensitivity in fish tissues.

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

2.6. Non-destructive PCR protocols.

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

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

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

3. RESULTS

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

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

The specificity of the primers Fer-3 and Fer-4 was assayed by using 69 strains of *A. salmonicida* and 38 of other bacterial species. All the *A. salmonicida* strains yielded the expected amplification product of 422 bp under the conditions chosen for the PCR programme (Table 2, Fig. 1 and 2a). Non-specific amplifications were obtained for some *A. hydrophila* strains whereas no amplification products were observed for any of 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 accesion N^{o} 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 $x10^2$ bacterial cells per 100 mg
236 of tissue when seeding a pure bacterial suspension (Fig. 3b), and of 4.5-10 x 10^2 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 10^2 bacterial cells mL^{-1}
241 of mucus (Fig. 4a), and for blood samples the lowest value detected was 1 x 10^5 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 *fst*A 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 posseses 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

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

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

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

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322 **REFERENCES**

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324 Austin B., Austin, D., 2007. Bacterial fish pathogens. Diseases of farmed and wild fish.
325 4th Ed. Springer-Praxis Publishing Ltd., Chichester, UK, 552 pp.

326 Avendaño-Herrera, R., Magariños, B., Toranzo, A.E., Beaz, R., Romalde, J.L., 2004.
327 Species-specific polymerase chain reaction primer sets for the diagnosis of
328 *Tenacibaculum maritimum* infection. Dis. Aquat. Org. 62, 75-83.

329 Brown, L.L., Iwama, G.K., Evelyn, T.P.T., Nelson, W.S., Levine, R.P., 1994. Use of the
330 polymerase chain reaction (PCR) to detect DNA from *Renibacterium salmoninarum*
331 within individual salmonid eggs. Dis. Aquat. Org. 18, 165-171.

332 Byers, H.K., Gugkovs, N., Crane, M.S., 2002. PCR- based assays for the fish pathogen
333 *Aeromonas salmonicida*. I. Evaluation of the three PCR primer sets for detection and
334 identification. Dis. Aquat. Org. 49, 129-138.

335 Cipriano, R.C., Ford, L.A., Teska, J.D., Shachte, J.H., Petrie, C., Novak, B.M., Flint,
336 D.E., 1996. Use of non-lethal procedures to detect and monitor *Aeromonas*
337 *salmonicida* in potentially endangered or threatened populations of migrating and
338 post-spawning salmon. Dis. Aquat. Org. 27, 233-236.

339 Cunningham, C. 2002, Molecular diagnosis of fish and shellfish diseases: present status
340 and potential use in disease control. Aquaculture 206, 19-55.

341 Del Cerro, A., Marquez, I., Guijarro, J.A., 2002. Simultaneous detection of *Aeromonas*
342 *salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri*, three major fish
343 pathogens, by multiplex PCR. Appl. Environ. Microbiol. 68, 5177-5180.

344 Fernández, A.I., Perez, M.J., Rodriguez, L.A., Nieto, T.P., 1995. Surface phenotypic
345 characteristics and virulence of Spanish isolates of *Aeromonas salmonicida* after
346 passage through fish. Appl. Environ. Microbiol. 61, 2010-2012.

347 Gustafson, C.E., Thomas, C.J., Trust, J.T., 1992. Detection of *Aeromonas salmonicida*
348 from fish by using polymerase chain reaction amplification of the virulence surface
349 array protein gene. Appl. Environ. Microbiol. 58, 3816-3825.

350 Hiney, M., Smith, P.R., 1998. Validation of polymerase chain reaction-based detection
351 techniques for bacterial pathogens in the environment: Framework, problems and
352 posible solutions. Aquaculture 162, 41-68.

353 Høie, S., Dalsgaard, I., Aase, I.L., Heum, M., Thornton, J.M., Powell, R., 1999.
354 Polymerase chain reaction (PCR)-based typing analysis of atypical isolates of the
355 fish pathogen *Aeromonas salmonicida*. System. Appl. Microbiol. 22, 403-411.

356 Miyata, M., Inglis, V., Aoki, T. , 1996. Rapid identification of *Aeromonas salmonicida*
357 subsp. *salmonicida* by the polymerase chain reaction. Aquaculture 141, 13-24.
358 O´Brien, D., Mooney, J., Ryan, D., Powell, E., Hiney, M., Smith, P.R., Powell, R.,
359 1994. Detection of *Aeromonas salmonicida*, causal agent of furunculosis in salmonid
360 fish, from the tank effluent of hatchery-reared Atlantic salmon smolts. Appl.
361 Environ. Microbiol. 60, 3874-3877.
362 Ortega, C., Fernández, A.B., Muzquiz, J.L., Ania, S., Gimeno, O., 2005. Health risks
363 associated with the migration of Atlantic salmon (*Salmo salar* L.): an
364 epidemiological surveillance programme in Northerh Spain. Rev. Sci. Tech. 24, 887-
365 898.
366 Osorio, C.R., Toranzo, A.E., 2002. DNA-based diagnostics in sea farming. In:
367 Fingerman, M., Nagabhushanam, R. (Eds.), Recent advances in Marine
368 Biotechnology. Vol 7. Seafood and human health,. Science Publishers, Inc., Enfield
369 (NH), USA. pp. 253-310.
370 Pazos, F., Santos, Y., Macías, A.R., Núñez, S., Toranzo, A.E., 1993. Evaluation of
371 media for the successful culture of *Flexibacter maritimus*. J. Fish Dis. 19, 193-197.
372 Pearson, W. R., Lipman, D.J., 1988. Improved Tools for Biological Sequence
373 Comparison. Proc Natl Acad Sci 85, 2444-2448.
374 Ramos, M.F., 2006. Caracterizaçao bioquímica e molecular de estirpes de *Aeromonas*
375 *salmonicida* subsp. *salmonicida* isoladas em Portugal. M.Sc. Thesis. University of
376 Porto. Portugal.
377 Reece, R.J., Maxwell, A., 1991. DNA gyrase: structure and function. Crit. Rev.
378 Biochem. Mol. Biol. 26, 335-375.
379 Romalde, J.L., Toranzo, A.E., 2002. Molecular aproaches for the study and diagnosis of
380 salmonid estreptococcosis. In: C. Cunningham (Ed.) Molecular diagnosis of
381 salmonid diseases. Kluwer Academic Publishers, Dordrecht. The Netherlands. pp.
382 211-233.
383 Romalde, J.L., Magariños, B., Villar, C., Barja, J.L., Toranzo, A.E., 1999. Genetic
384 analysis of turbot pathogenic *Stretococcus parauberis* strains by ribotyping and
385 random amplified polymorfic DNA. FEMS Microbiol. Lett. 179, 297-304.
386 Taylor, P.W., Winton, J.R., 2002. Optimization of nested polymerase chain reaction
387 assay for identification of *Aeromonas salmonicida*, *Yersinia ruckeri*, and
388 *Flavobacterium psychrophilum*. J. Aquat. Anim. Health 14, 216-224.
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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 *Scopthalmus 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**
Aeromonas bestiarum	CECT 4227	CECT	NSA[b]
Aeromonas hydrophila	020116-03	Laboratory collection	NSA
A. hydrophila	055102-04	Laboratory collection	NSA
A. hydrophila	X.P. Alev.	Laboratory collection	–
A. hydrophila	TB 14.1	Laboratory collection	–
A. hydrophila	061002-01	Laboratory collection	NSA
A. hydrophila	PC 522.1	Laboratory collection	NSA
A. hydrophila	RPM 747.1	Laboratory collection	–
A. hydrophila	010530-01	Laboratory collection	NSA
A .hydrophila	B 32	Laboratory collection	NSA
A. hydrophila	TW 40.1	Laboratory collection	–
A. hydrophila	PC 521.2	Laboratory collection	NSA
A. hydrophila	053102-04	Laboratory collection	NSA
A. sobria	P33	Laboratory collection	–
A. media	ATCC 33907	ATCC	NSA
A. caviae	P322	Laboratory collection	–
Vibrio harveyi	RA 58.2	Laboratory collection	–
V. anguillarum	R 72	Laboratory collection	–
V. anguillarum	TM 20.1	Laboratory collection	–
V. splendidus	ATCC 33125	ATCC	–
V. scopthalmi	CECT 4638	CECT	–
V. furnisii	CECT 4203	CECT	–
V. natriegens	ATCC 14048	ATCC	–
V. tubiashi	ATCC 19106	ATCC	–
V. nereis	ATCC 25917	ATCC	–
V. ordalii	NCIMB 2107	NCIMB	–
V. ficheri	NCIMB 1274	NCIMB	–
V. vulnificus	ATCC 27562	ATCC	–
Yersinia ruckeri	TW 330	Laboratory collection	–
Photobacterium damselae	P12	Laboratory collection	–
Tenacibaculum maritimus	PC 560.1	Laboratory collection	–
Marinomonas vaga	NCIMB 1962	NCIMB	–
Oceanomonas doudoroffi	NCIMB 1965	NCIMB	–
P. aeruginosa	ATCC 27853	ATCC	–
P. fluorescens	ATCC 13525	ATCC	–
P. anguilliseptica	TW-P1	Laboratory collection	–
Pseudomonas sp.	RP 20.1	Laboratory collection	–
Lactococcus garvieae	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
*fst*A	Fer-1 / Fer-2	5´CGCTCGCCCATCCCCCTCTG 3´ (f) 5´GCCCCTTGCACCCCCACCATT 3´ (r)	110 - 129 561 - 541	452	This work
*fst*A	Fer-3 / Fer-4	5´CGGTTTTGGCGCAGTGACG 3´ (f) 5´AGGCGCTCGGGTTGGCTATCT 3´ (r)	1683 -1701 2104 - 2084	422	This work
*fst*A	Fer-5 / Fer-6	5´AAGGGCCCGTCCGTGTTGCTCA3´ (f) 5´ATACGGCTGCCCTGGTGTCATT 3´ (r)	886 - 907 1348 - 1327	463	This work
*gyr*B	Asg-1 / Asg-2	5´TGGCATGGAACATTCCTCCT 3´ (f) 5´GTCGCCTGCTTTTTCCAGCA 3´ (r)	726 - 746 1485 - 1465	760	This work
*vap*A	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.

Figure Legends

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

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

Fig. 3. Sensitivity of the PCR protocol with Fer-3 and Fer-4 using purified DNA from the isolate *A. salmonicida* ATCC 14174 (A), DNA extracted from fish tissues seeded with different concentrations of *A. salmonicida* (ATCC 14174) in pure culture (B) and with mixed cultures of *A. salmonicida* (ATCC 14174), *A. hydrophila* (RPM 747.1) and *V. anguillarum* (R72)(C). Lanes in A: M, Amplisize Molecular Ruler (50-2000 bp ladder, Sigma); 1 to 9, DNA extracted from serial decimal dilutions of the isolate ATCC 14174, ranging from 2 x 10^8 (Lane 1) to 2 x 10^0 (Lane 9) cells mL^{-1}. Lanes in B: M, Amplisize Molecular Ruler (50-2000 bp ladder, Sigma); 1 to 6, amplified DNA corresponding to 6 x 10^5 (Lane 1) to 6 x10^0 (Lane 6) cells $100mg^{-1}$, 7, negative control (unseeded tissue). Lanes in C: M, Amplisize Molecular Ruler (50-2000 bp ladder, Sigma); 1, negative control (unseeded tisssues); 2 to 7, amplified DNA corresponding to 4.5 x 10^6 to 4.5 x10^1 cells $100mg^{-1}$. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified product for *A. salmonicida*.

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

Figure 1

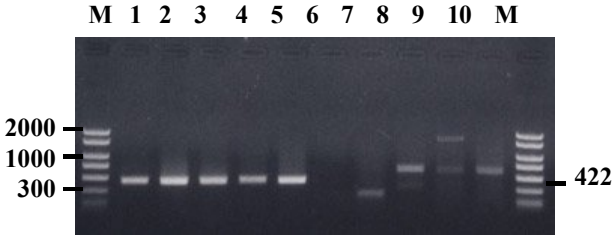

Fig. 1.- Beaz-Hidalgo et al.

Figure 2

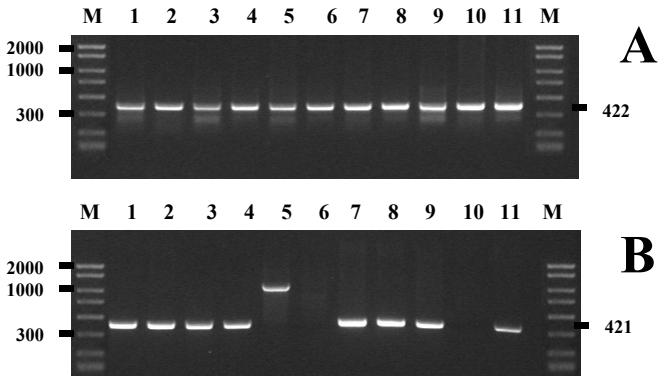

Fig. 2.- Beaz-Hidalgo et al.

Figure

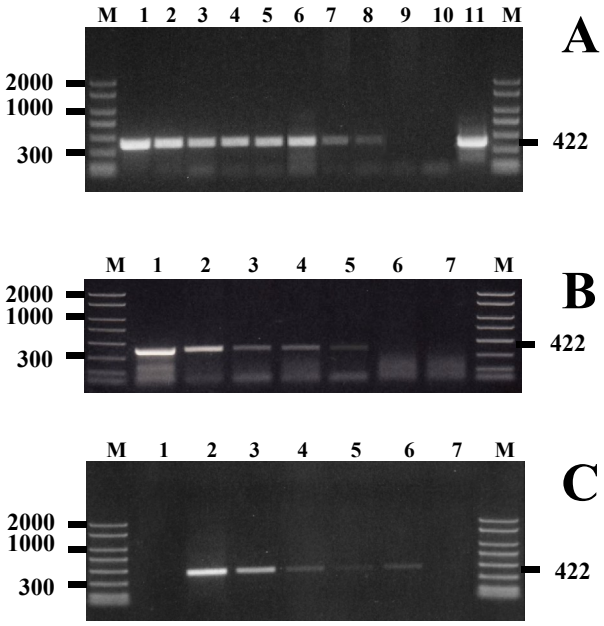

Fig. 3.- Beaz-Hidalgo et al.

Figure

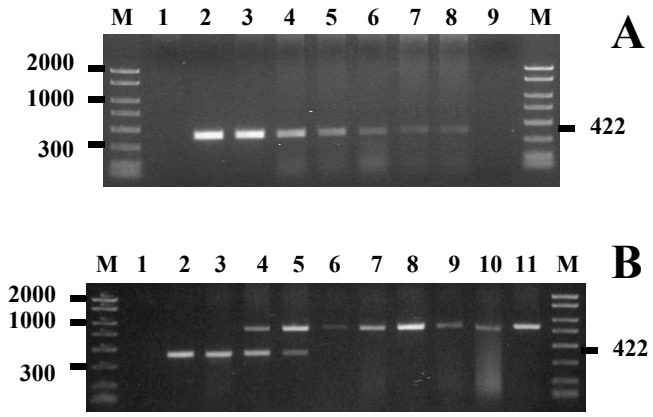

Fig. 4.- Beaz-Hidalgo et al.