

Molecular Detection and Characterization of Furunculosis and Other *Aeromonas* Fish Infections

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1. Introduction

Species of the genus *Aeromonas*, which inhabit aquatic environments, can produce septicaemia and ulcerative and hemorrhagic fish diseases, including furunculosis, which result in mass death and important economic losses in the aquaculture sector (Austin & Austin, 2007; Beaz-Hidalgo et al., 2010; Bernoth et al., 1997; Gudmundsdóttir & Björnsdóttir, 2007; Noga, 2010; Wiklund & Dalsgaard, 1998). The species *Aeromonas salmonicida* (with 5 subspecies: *salmonicida*, *masoucida*, *smithia*, *achromogenes* and *pectinolytica*) and *Aeromonas hydrophila* have classically been considered the most important *Aeromonas* fish pathogens, and the subspecies *salmonicida* is thought to be the causal agent of furunculosis, a disease that was reported more than a century ago to affect trout, and later to affect other salmonids and fish species (Bernoth et al., 1997; Goodwin & Merry, 2009; Han et al., 2011; Noga, 2010; Wiklund & Dalsgaard, 1998). Nowadays, furunculosis has a worldwide distribution, having been reported in Scotland, France, Norway, Iceland, Spain, United States, Canada, Japan, Chile and Australia. The fact that many strains isolated from diseased fish do not fit the described characteristics defined for *A. salmonicida* subsp. *salmonicida* or for furunculosis has led to the terms 'atypical strains' and 'atypical furunculosis' to be introduced when they are attributed to other *A. salmonicida* subspecies, other *Aeromonas* species or when furunculosis occurs in fish other than salmonids (Wiklund & Dalsgaard, 1998). This causes confusion because the term 'atypical' is applied in different ways by the authors.

Publications such as the specific furunculosis monography published in 1997 by Bernoth et al., chapters dealing with this infection in specific aquaculture books (Austin & Austin, 2007; Hiney & Olivier, 1999; Noga, 2010) and reviews on atypical and typical furunculosis (Wiklund & Dalsgaard, 1998) and their treatment (Gudmundsdóttir & Björnsdóttir, 2007) among others, all reflect the importance to the aquaculture sector of *Aeromonas* infections. Several PCR methods have been designed and evaluated comparatively for the fast detection and identification of typical and atypical *A. salmonicida* from infected fish tissue and the introduction of reliable molecular *Aeromonas* identification methods have enabled new species (*Aeromonas tecta* and *Aeromonas piscicola*) to be discovered and/or other known species associated to fish disease to be recognized i.e. *Aeromonas bestiarum*, *Aeromonas sobria*, *Aeromonas encheleia*, *Aeromonas veronii*, *Aeromonas eucrenophila* and *Aeromonas media* (Beaz-Hidalgo et al., 2010; Kozińska, 2007; Li & Cai, 2011; Nawaz et al., 2006; Soriano-Vargas et al.,

2010). These species would have remained masked as, mainly, *A. salmonicida* or *A. hydrophila* if biochemical identification had only been applied and would have misrepresented the real prevalence and/or diversity of the species implicated in fish infections (Beaz-Hidalgo et al., 2010; Figueras et al., 2011b).

This chapter on *Aeromonas* fish infections presents the most recent information on the taxonomy and identification of these microorganisms derived from the application of molecular techniques together with a detailed review of the currently available PCR methods that have been designed for the detection or characterization of typical or atypical *A. salmonicida* or other *Aeromonas* species in, mainly, fish tissue and water during outbreaks or regular preventive monitoring. Preventive strategies for the control of infectious fish diseases, such as those produced by *Aeromonas* are a constant challenge, and need to be regularly reviewed in order to recognize dynamic changes associated to these diseases, which may be caused both by the emergence of new pathogenic species (i.e. the recently discovered *A. piscicola* and *A. tecta*) or by environmental factors. Regarding the latter, climate change is considered to play a role in the appearance and impact of furunculosis (Tam et al., 2011) and is therefore another aspect addressed in this chapter.

2. The genus *Aeromonas*

The genus *Aeromonas* belongs to the class *Gammaproteobacteria*, order *Aeromonadales* and family *Aeromonadaceae* (Martin-Carnahan & Joseph, 2005). *Aeromonas* species are widely distributed in aquatic environments and are isolated from water, healthy or diseased fish, food products, animal and human faeces and other clinical and environmental samples (Figueras, 2005; Janda & Abbot, 2010). The first description of an *Aeromonas* species dates back to 1891, when Stainer described the bacteria *Bacillus hydrophilus fuscus* (now *A. hydrophila*) isolated from diseased frogs (Martin-Carnahan & Joseph, 2005). A few years later in 1894 Emmerich and Weibel described the species *Bacillus de Forellenseuche* (later *Bacillus salmonicida*, now *A. salmonicida*) isolated from diseased trout (Martin-Carnahan & Joseph, 2005). The formal description of the genus was made by Stainer in 1943, and in the 1970s *Aeromonas* species were classified into 2 groups on the basis of their growth temperature, motility and pigment production (Martin-Carnahan & Joseph, 2005). One group comprised mesophilic strains able to grow at 37°C, motile and non-pigmented, mainly associated with human clinical infections and represented by *A. hydrophila*. The other group comprised psychrophilic strains (optimum growth at 22-28°C), non-motile and pigmented, which were mainly fish pathogens represented by *A. salmonicida*. In the 1980s, DNA-DNA hybridization assays allowed the differentiation of various genospecies or hybridization groups (Martin-Carnahan & Joseph, 2005).

In the last edition of Bergey's Manual (Martin-Carnahan & Joseph, 2005) the genus, that previously belonged to the family *Vibrionaceae* was placed in its own independent family *Aeromonadaceae*, it comprised 14 species i.e. *A. hydrophila* (with 2 subspecies: *hydrophila* and *ranae*), *A. bestiarum*, *A. salmonicida* (with 5 subspecies: *salmonicida*, *masoucida*, *smithia*, *achromogenes* and *pectinolytica*), *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii*, *A. encheleia*, *Aeromonas jandaiei*, *Aeromonas schubertii*, *Aeromonas trota*, *Aeromonas allosaccharophila*, and *Aeromonas popoffii*. Also some species were synonymised with previously recognized species such as *A. ichthiosmia* and *A. culicicola* with *A. veronii* and *A. enteropelogenes* with *A. trota*. Since then, the genus has expanded rapidly with the addition of 11 new species

(*Aeromonas simiae*, *Aeromonas molluscorum*, *Aeromonas bivalvium*, *A. tecta*, *Aeromonas aquariorum*, *A. piscicola*, *Aeromonas fluviialis*, *Aeromonas taiwanensis*, *Aeromonas sanarellii*, *Aeromonas diversa* and *Aeromonas rivuli*) and today the genus comprises 25 validated species (Figueras et al., 2011b).

3. Furunculosis and other *Aeromonas* fish infections

Furunculosis is one of the oldest known important diseases in aquaculture first documented by Emmerich and Weibel in 1894 when they observed furuncles or ulcer lesions on the trout's skin. These characteristic ulcerated lesions are those that give rise to the name of the disease attributed to infections produced by *Bacillus de Forellenseuche*, now known as *A. salmonicida* (Austin & Austin, 2007; Bernoth et al., 1997; Martin-Carnahan & Joseph, 2005). In reality, furunculosis was the first fish infection for which the 'Koch postulates' were demonstrated more than a century ago (Austin & Austin, 2007; Bernoth et al., 1997). It was thought originally that the infection affected only salmonids, but it soon became evident that it was distributed worldwide and affected many other marine and freshwater fish species, such as the Atlantic cod (*Gadus morhua*), halibut (*Hyppoglossus hypoglossus*), turbot (*Scophthalmus maximus*), lamprey (*Petromyzon marinus*), carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and eel (*Anguilla anguilla*) among others (Austin & Austin, 2007; Bernoth et al., 1997; Godoy et al., 2010; Goldschmidt-Clermont et al., 2009; Noga, 2010; Wiklund & Dalsgaard, 1998).

Lethargy, lack of appetite, or skin hyperpigmentation can be the first signs of the infection and it may show other clinical manifestations such as the presence of the typical furuncles or ulcers, exophthalmia (swelling of the eyes), septicaemia, petequias (small haemorrhagic lesions due to broken blood capillaries), anemia, ascitis and haemorrhages in the muscle, gills, fins, nares, vent and internal organs (Austin & Austin, 2007; Bernoth et al., 1997; Hiney & Olivier, 1999; Wiklund & Dalsgaard, 1998). On the other hand, fish affected by furunculosis do not always show all these clinical symptoms and may even not show the typical furuncles or skin ulcers (Noga, 2010).

Many *A. salmonicida* carrier fish that do not show external lesions or clinical signs of the disease but that are indeed able to shed the microorganism (10^5 - 10^6 CFU per fish/h) and to develop the disease under conditions of stress (i.e. increase in water temperature, poor water quality, etc), producing an epidemic outbreak have been reported (Bernoth et al., 1997; Gustafson et al., 1992; Hiney & Olivier, 1999; Noga, 2010; Wiklund & Dalsgaard, 1998). It has been estimated that up to 80% of cultivated trout may carry *A. salmonicida*, thus increasing the likelihood of transmission to susceptible fish (Gustafson et al., 1992). These covertly infected fish (or carriers) play an important role in the epidemiology of the disease, therefore early detection of the pathogen is very important. Several PCR detection methods have been developed for the detection of *A. salmonicida* (see Sections 4.2.2-4.2.5, Tables 1-5), whose application is essential for an effective disease control (Altinok et al., 2008; Byers et al., 2002a, b; Gustafson et al., 1992; Onuk et al., 2010).

Diseases caused by atypical strains of *A. salmonicida* (those that do not belong to the subsp. *salmonicida*) are referred to as "atypical furunculosis", although this terminology is also applied to infections produced in non-salmonids. Furthermore, more specific names have been used to refer to infections affecting specific fish, i.e. goldfish ulcer disease, carp

erythrodermatitis, and flounder ulcer disease (Austin & Austin, 2007; Noga, 2010; Wiklund & Dalsgaard, 1998). Despite some authors have tried to describe specific clinical signs for each of these named pathologies (Austin & Austin, 2007; Noga, 2010), in practice they are indistinguishable from those associated to furunculosis or other ulcerative diseases or septicemia caused by other motile *Aeromonas* species. The latter has also been named 'motile *Aeromonas* septicemia' and is considered a clear example of a stress-induced disease mainly affecting freshwater fish. Although motile species also inhabit brackish water, their prevalence decreases with increasing salinity (Austin & Austin, 2007; Noga, 2010). These infections are mainly attributed to *A. hydrophila*, classically considered the most important species after *A. salmonicida*. However, the importance of the species *A. hydrophila* is overestimated because biochemical identification systems erroneously identify up to 70-80% of the strains of *Aeromonas* as belonging to this species, when in fact they are many different species when identified by molecular methods (Beaz-Hidalgo et al., 2010; Figueras, 2005; 2011b; Soler et al., 2003a). Prevalent motile species associated to diseased fish following molecular identification include *A. veronii*, associated with catfish, *A. sobria* with tilapia and trout, *A. hydrophila* with trout, *A. bestiarum* with carp and the recently described *A. piscicola* recovered from diseased salmonids and turbot (Beaz-Hidalgo et al., 2010; Kozłowska, 2007; Li & Cai, 2011; Martino et al., 2011; Nawaz et al., 2006; Soriano-Vargas et al., 2010). Other motile species, such as *A. encheleia*, *A. allosaccharophila*, *A. jandaei*, *A. media*, *A. eucrenophila*, *A. aquariorum* and *A. tecta* have also been recovered from healthy or diseased fish, though less often (Beaz-Hidalgo et al., 2010; Kozłowska, 2007).

Typical or atypical furunculosis and motile *Aeromonas* septicemias all produce similar clinical signs that are also common to those observed in other fish systemic diseases caused by other bacterial and viral pathogens (Austin & Austin, 2007; Bernoth et al., 1997; Noga, 2010; Wiklund & Dalsgaard, 1998). Therefore, a definitive diagnosis requires the isolation, culture and identification of the bacteria recovered from the lesions or internal organs of the diseased fish and/or from the dead fish. Molecular PCR methods are available (Tables 1-5) that specifically detect *A. salmonicida* without the need for culturing. Care is needed in the case of covertly infected fish because false negatives can be obtained. Some authors recommend using a pre-enrichment step before the PCR detection or to use both culturing and PCR detection in parallel (Byers et al., 2002a; Gustafson et al., 1992).

3.1 Isolation of *Aeromonas* sp. from diseased fish

Recognizing a bacterial fish pathogen requires their analysis in several organs (mainly kidney, but also spleen, skin, and ovarian fluid in breeding females) of a significant number of living fish (Byers et al., 2002b; Noga, 2010). Between 4 and 10 diseased fish are recommended to be sampled for detecting the pathogen, and between 10 and 60 fish in a population where there is a low mortality rate or the fish are apparently healthy (Noga, 2010). Detecting the pathogen in mucus, blood or faeces is also recommended because these samples do not require the fish to be sacrificed (Beaz-Hidalgo et al., 2008; Byers et al., 2002b; Gustafson et al., 1992; Kulkarni et al., 2009). When searching for asymptomatic carriers, the kidney and intestine are the recommended organs to sample. Skin and gills can also be cultured, but *A. salmonicida* may be uncovered by the presence of other dominant bacteria (Noga, 2010). False negatives in carrier fish are common since the bacteria are present in low concentrations (Byers et al., 2002b; Gustafson et al., 1992; Noga, 2010). More asymptomatic

carriers can be detected by including either a pre-enrichment step before the PCR analysis, as commented before (Byers et al., 2002b; Gustafson et al., 1992) or by inducing stress using the approach named 'stress induced furunculosis' (SIF). The latter requires an intraperitoneal injection of glucocorticoids (which generates immunosuppression) and then exposure of the fish to a heat shock (raising the temperature of the water to 18°C for 14 days). This favours bacterial proliferation and the appearance of clinical signs, and will also increase the recovery rate of the bacteria (Austin & Austin, 2007; Bernoth et al., 1997; Byers et al., 2002b; Hiney & Olivier, 1999; Noga, 2010).

Non-selective culture media used for the isolation of *Aeromonas* strains include trypticase soy agar (TSA), brain heart infusion agar (BHI) or Columbia blood agar (Austin & Austin, 2007; Bernoth et al., 1997; Hiney & Olivier, 1999). More selective media used for the recovery of *A. salmonicida* include the Furunculosis agar (tryptone, yeast extract, L-tyrosine and NaCl), TSA or BHI supplemented with L-tyrosine (0.1%) or TSA supplemented with Coomassie brilliant blue (0.01%). The latter method was designed for detecting strains that have the A-layer (or S-layer), one of the oldest known outer membrane proteins associated with *Aeromonas* infection in fish, which is implicated in the resistance against the host complement system and enables the bacteria to adhere to the host proteins, facilitating colonization (Gustafson et al., 1992). The Coomassie brilliant blue attaches to the A-layer and the colonies are then seen as deep blue in comparison with white or light blue negative A-layer colonies (Austin & Austin, 2007; Bernoth et al., 1997). However, this method is not specific for *A. salmonicida*, since other *Aeromonas* or other bacterial (*Pseudomonas*, *Pasteurella*, *Corynebacterium*) species pathogenic to fish also have the A-layer and are able to grow in this culture media. Once the bacteria are isolated they can be identified by serological, phenotypic or molecular methods, the latter two being discussed in the next section.

4. Identification of *Aeromonas* species

This section describes the established phenotypic characteristics that differentiate the genus *Aeromonas* from other related genera, the limitations of the conventional and miniaturised biochemical identification systems for differentiating all the species, as well as the new panorama derived from the application of molecular techniques.

4.1 Phenotypic identification with conventional methods and miniaturised commercial systems

Aeromonas species are phenotypically characterized as Gram-negative bacilli, with cytochrome oxidase generally positive, an ability to grow at 0% of NaCl but not at 6%. They do not produce acid from inositol, are able to ferment glucose and most are resistant to the vibriostatic agent O/129 2,4-diamino-6,7-diisopropyl-pteridine-phosphate. The genus *Aeromonas* can be differentiated from other closely-related genera like *Plesiomonas* by its fermentation of inositol and its resistance to the vibriostatic agent, and from *Vibrio* by its ability to grow at 0% but its inability to grow at 6% of NaCl (Martin-Carnahan & Joseph, 2005). Optimum growth temperature for *Aeromonas* species is 22-37°C, except for some strains of *A. salmonicida*, which is 22-25°C (Martin-Carnahan & Joseph, 2005).

As indicated previously, the species *A. salmonicida* includes 5 subspecies (*A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *smithia*, *A.*

salmonicida subsp. *masoucida* and *A. salmonicida* subsp. *pectinolytica*), which are practically impossible to differentiate using phenotypic or molecular methods. All of them, except the subspecies *pectinolytica* have been implicated in fish pathology (Austin & Austin, 2007; Goldschmidt-Clermont et al., 2009; Han et al., 2011; Noga, 2010; Wiklund & Dalsgaard, 1998). Being unable to assign strains implicated in fish disease to any of the subspecies *masoucida*, *smithia*, *achromogenes* or *pectinolytica* using biochemical characteristics, they have been termed “atypical *A. salmonicida*” to differentiate them from the “typical *A. salmonicida*”, a term restricted to the subspecies *salmonicida* (Beaz-Hidalgo et al., 2008; Goodwin & Merry, 2009; Wiklund & Daalsgard 1998). “Typical *A. salmonicida*” strains are psychrophilic, non-motile and produce brown pigment. In contrast, the “atypical *A. salmonicida*” are a more heterogeneous group with different phenotypic features and are usually isolated from non-salmonid fish (Noga, 2010). Among the “atypical *A. salmonicida*” strains, there is a group of strains that show a mesophilic behaviour (growing at 37°C), are motile and do not produce pigment and that have been also identified from human clinical samples (Figueras, 2005; Martínez-Murcia et al., 2005). Some of the mentioned characteristics (motility and mesophilic behaviour) are also phenotypic traits of *A. salmonicida* subsp. *pectinolytica* (Pavan et al., 2000). To distinguish these mesophilic *A. salmonicida* strains from other mesophilic species like *A. bestiarum* or *A. piscicola* is practically impossible when using biochemical characteristics or the sequences of the 16S rRNA gene (Beaz-Hidalgo et al., 2010; Figueras et al., 2011b; Martínez-Murcia et al., 2005). However, these species can be differentiated with sequences of the housekeeping gene *rpoD* (Beaz-Hidalgo et al., 2010).

Numerous biochemical schemes have been proposed for the characterization of *Aeromonas* species, but they mainly discriminate three big phenotypic groups i.e. the “*A. hydrophila*” complex (including *A. hydrophila*, *A. bestiarum* and *A. salmonicida* and also latter *A. popoffii*), the “*Aeromonas caviae*” complex (including *A. caviae*, *A. media* and *A. eucrenophila*) and “*Aeromonas sobria*” complex (including *A. sobria*, *A. veronii*, *A. jandaei* and *A. trola*) (Abbott et al., 1992; 2003; Borrell et al., 1998; Kozińska et al., 2002; Martin-Carnahan & Joseph, 2005; Martínez-Murcia et al., 2005). In the descriptions of new *Aeromonas* species, the mandatory criteria of identifying differential phenotypic tests from other existing *Aeromonas* species has been fulfilled (Figueras et al., 2011b and references therein). However, biochemical tests may show intra-species variability either due to the variable nature of the bacterial phenotypic characters or due to a lack of reproducibility of the tests when they are carried out under different laboratory conditions, such as temperatures, etc. (Figueras et al., 2011b). Biochemical identifications in fact lack precision and tend to generate a lot of inconsistent results in comparison to those obtained by molecular methods (Beaz-Hidalgo et al., 2010; Figueras et al., 2011b; Kozińska et al., 2002, Kozińska, 2007; Nawaz et al., 2006).

Miniaturized commercial identification systems, such as API (20E, 20NE), Vitek, Biolog GN Microplates, BBL Crystal or MicroScan Walk/Away are also routinely used in ichthyopathology laboratories, but are not always able to identify *Aeromonas* species precisely (Figueras, 2005; Joseph & Carnahan, 1994; Kozińska et al., 2002; Park et al., 2003; Soler et al., 2003a). The mentioned systems normally tend to identify all *Aeromonas* strains as *A. hydrophila* (Figueras, 2005; Soler et al., 2003b). Some other authors have also reported poor results from the API systems and discrepancies with traditional tube testing when identifying *Aeromonas* strains isolated from fish (Godoy et al., 2010; Han et al., 2011; Joseph & Carnahan, 1994; and references therein). Some methods may even misidentify *Aeromonas* strains as *Vibrio* species (Park et al., 2003; Soler et al., 2003b). To avoid this confusion,

Chacón and colleagues designed a specific probe based on a fragment of the glycerophospholipid-cholesterol acyltransferase (GCAT) gene that is able to hybridize with all *Aeromonas* species but not with strains from other genera (Chacón et al., 2002).

4.1.1 Discrepancies between biochemical and molecular identification methods

Because phenotypic characterization can give imprecise results, one cannot guarantee that a strain has been correctly identified at the species level, especially for complex genera like *Aeromonas*, if it has not been verified later using reliable molecular methods (Figueras et al., 2011a). Using biochemical and genetic methods in parallel for species identification has allowed big discrepancies to be highlighted and the characteristic phenotypic traits that might be responsible for the confusion to be discovered (Beaz-Hidalgo et al., 2010; Kozińska, 2007; Nawaz et al., 2006).

In a recent study, Nawaz et al. (2006) demonstrated that with the Vitek-GNI system 81 strains isolated from the intestines of catfish (*Ictalurus punctatus*) collected from different geographical regions of United States were identified as *A. hydrophila* (n=23), *A. trota* (n=7), *A. veronii* (n=42), *A. caviae* (n=6) and *A. jandaei* (n=3). However, when they were evaluated with the restriction fragment length polymorphism of the 16S rRNA gene (16S rDNA-RFLP) method proposed for *Aeromonas* identification (Borrell et al., 1997; Figueras et al., 2000), all the 81 strains were identified as *A. veronii* (Nawaz et al., 2006). In this case, the errors in phenotypic identification were masking the importance that the species *A. veronii* may have in catfish pathology.

In another recent study, Beaz-Hidalgo et al. (2010) used phenotypic and genetic methods (16S rDNA-RFLP and sequencing of the *rpoD* gene) to retest 119 *Aeromonas* strains recovered from diseased fish and shellfish that had been biochemically identified in a routine ichthyopathology laboratory. They found that, of the strains considered to belong to the genus *Aeromonas* using biochemical methods, 24.4% (29/119) did not belong when using the genus-specific PCR identification method based on the GCAT gene, which is specific for the genus *Aeromonas* as previously explained (Chacón et al., 2002). Sequencing the 16S rRNA gene identified these strains as belonging to the genera *Pseudomonas* and *Vibrio*. Considering only the 86 *Aeromonas* strains isolated from diseased fish, the species recognised by biochemical identification methods were: *A. hydrophila* (n=63), *A. salmonicida* (n=12), *A. sobria* (n=2), *A. veronii* (n=2), *A. media* (n=1), *A. trota* (n=1), *A. bestiarum* (n=1) and 4 strains could not be assigned to any known species (*Aeromonas* sp.). However, 66.3% (57/86) of them had been incorrectly identified phenotypically when compared with the genetic results that showed that the order of prevalence was as follows: *A. sobria* 25.6% (22/86), *A. hydrophila* 17.4% (25/86), *A. salmonicida* 17.4% (15/86), *A. bestiarum* 16.3% (14/86), *A. piscicola* 11.6% (10/86), *A. media* 7% (6/86), *A. eucrenophila* 2.3% (2/86), *A. encheleia* 1.2% (1/86) and *A. tecta* 1.2% (1/86) (Beaz-Hidalgo et al., 2010). The strains of the species *A. veronii* (n=2) and *A. trota* (n=1) therefore belonged to totally different species. For instance, the two *A. veronii* belonged to *A. sobria* and *A. piscicola*, while *A. trota* was *A. sobria* and the 4 strains of *Aeromonas* sp. belonged to *A. sobria* (2 strains), *A. media* and *A. bestiarum*. Furthermore, genetic identification revealed that 50 of the 64 phenotypically identified *A. hydrophila* strains did in fact belong to 8 other *Aeromonas* species, including 17 strains belonging to *A. sobria* and 6 strains to new species *A. piscicola* (Beaz-Hidalgo et al., 2009; 2010). This study clearly highlighted once more that there was a false importance attributed to the species *A.*

hydrophila as the most prevalent (Beaz-Hidalgo et al., 2010; Figueras, 2005; Figueras et al., 2011b; Soler et al., 2003b). Regarding *A. salmonicida*, 85.7% (12/14) of the strains that showed to be psychrophilic, non motile and pigment producers had been correctly identified. The two strains misidentified phenotypically as *A. hydrophila* showed mesophilic behaviour (i.e. were motile and did not produce pigment). Looking at the most prevalent *Aeromonas* species by fish host, it was found that *A. sobria* (15/22 strains) and *A. hydrophila* (8/15 strains) were more associated with trout, *A. salmonicida* with Atlantic salmon and turbot (6/15 strains each) and *A. piscicola* (5/10 strains) and *A. bestiarum* (8/14 strains) with Atlantic salmon (Beaz-Hidalgo et al., 2010). The only strain of the species *A. tecta* was isolated from bogue (*Chondrostoma comun*), this being the first report in this kind of fish and the second report from fish since its recent description from trout in 2008 (Beaz-Hidalgo et al., 2010 and references therein).

These results gave evidence of a greater diversity of *Aeromonas* species involved in fish pathology than was first thought and that the species *A. salmonicida* or *A. hydrophila* were not the most prevalent.

Despite the known limitations of phenotypic identification methods, they are still being used in ichthyopathology laboratories, which contributes to an underestimation of the true diversity of *Aeromonas* species associated with diseased fish (Beaz-Hidalgo et al., 2010).

4.2 Molecular identification

This section describes the above-mentioned molecular methods used for identifying *Aeromonas* in fish pathology (i.e. 16S rDNA-RFLP, the sequences of the 16S rRNA gene or other housekeeping genes) as well as the different PCR detection and identification methods that target typical and atypical *A. salmonicida*.

4.2.1 The 16S rRNA and the housekeeping genes

The 16S rRNA gene is essential to bacteria and has been used as a specific molecular marker for their identification (Alperi et al., 2008 and references therein; Martínez-Murcia et al., 1992). An RFLP method based on this gene was developed by our group to differentiate all the *Aeromonas* species described up to 2000 (Borrel et al., 1997; Figueras et al., 2000) and has proven to be useful to different authors who have identified the strains recovered from diseased fish (Beaz-Hidalgo et al., 2010; Kozińska et al., 2002; Kozińska, 2007; Nam & Joh, 2007; Nawaz et al., 2006; Soriano-Vargas et al., 2010). However, this method is not able to differentiate closely related species that have an equal or almost equal 16S rRNA gene sequence because it produces the same RFLP pattern for all of them. This is shown in the case of the new species *A. piscicola*, which has the same RFLP pattern as *A. salmonicida* and *A. bestiarum* whose 16S rRNA sequences share 99.8-100% similarity (Beaz-Hidalgo et al., 2010; Figueras et al., 2011b). The same is true with *A. caviae* and *A. aquariorum*, which show a 99.8% similarity and the same RFLP pattern (Figueras et al., 2009, 2011b). The latter, i.e. *A. aquariorum*, was isolated originally from ornamental fish, but has not so far been isolated again from other fish. The 16S rDNA-RFLP method is also not useful for the 8% of *Aeromonas* strains that show mutations on the 16S rRNA gene in the targeted region of the endonucleases used, because these produce a different pattern from the one expected for the species (Alperi et al., 2008).

In the genus *Aeromonas* the sequences of the 16S rRNA gene are not very useful tools for identification because only a few distant species can be well discriminated, but it is useful for confirming whether or not the strains under analysis belong to the genus (Figueras et al., 2011b; Han et al., 2011).

As a complementary tool to the 16S rDNA-RFLP for identifying all *Aeromonas* species, we have introduced the use of housekeeping genes that codify essential proteins for the survival of the bacteria (Figueras et al., 2011b; Soler et al., 2004; Yáñez et al., 2003). In reality, the analysis of 5 housekeeping genes is one of the methods used in the description of new bacterial taxa that has been recommended by the *ad hoc* committee for the re-evaluation of the species definition in bacteriology (Stackebrandt et al., 2002). The first housekeeping genes described for the identification of *Aeromonas* were the *gyrB* gene, which codifies for the subunit B of the DNA gyrase (Yáñez et al., 2003) and the *rpoD* gene, which encodes the σ^{70} factor of the RNA polymerase (Soler et al., 2004). Nowadays the cost of sequencing has fallen considerably being much cheaper to use external services that provide the sequences from a given extracted DNA than to perform the sequencing at your own laboratory. Therefore we recommend sequencing the *rpoD* or *gyrB* gene to establish the identity of the isolated strain because it is a faster and more reliable identification approach than the 16S rDNA-RFLP (Figueras et al., 2011b). However, misinterpretations may still occur when comparing the sequences obtained with those available for all the species of the genus that are held at the GenBank, especially if sequences are too short or of poor quality. Furthermore the database also contains wrongly labelled strains, so comparison should always be made with the sequences of type strains (Figueras et al., 2011b). We also recommend to deposit the new sequences at the GenBank for further broadening of the existing database. Figure 1 illustrates a phylogenetic tree constructed with the *rpoD* sequences of the type strains of the 25 species that include at present the genus and several *Aeromonas* strains isolated from diverse diseased fish studied in our laboratory.

Other housekeeping genes that have been used in *Aeromonas* are the *rpoB*, which codifies for the β subunit of the RNA polymerase (Küpfer et al., 2006), the *dnaJ*, which codifies a thermal shock protein (Nhung et al., 2007), the *recA*, which codifies a protein involved in DNA repair (Sepe et al., 2008) and the *cpn60*, which codifies the chaperone Cpn60 type I that is involved in protein assembly (Miñana-Galbis et al., 2009). A Multilocus Phylogenetic Analysis (MLPA) of the genus *Aeromonas* has recently been established using the concatenated information derived from the sequences of 7 housekeeping genes: *gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX*, and *atpD* (Martínez-Murcia et al., 2011). The resulting phylogenetic tree (4705 bp) agreed with the taxonomy of the genus as recognized to date, showing that the MLPA is a robust way of identifying unequivocally all the species. The MLPA is also called Multilocus Sequence Analysis (MLSA) by some authors, which derives from the original name Multilocus Sequence Typing (MLST) (Martínez-Murcia et al., 2011). Nowadays, MLST schemes are recognised to be the best way of establishing the epidemiological relationships among isolates using the sequences of internal fragments of multiple housekeeping genes (approximately 450-500 bp of 7 genes). For each gene the sequences of different strains are compared and each unique sequence is assigned a specific number. The strains that show the same numbers assigned to all genes belong to the same sequence type (ST) which is also identified by a specific number.

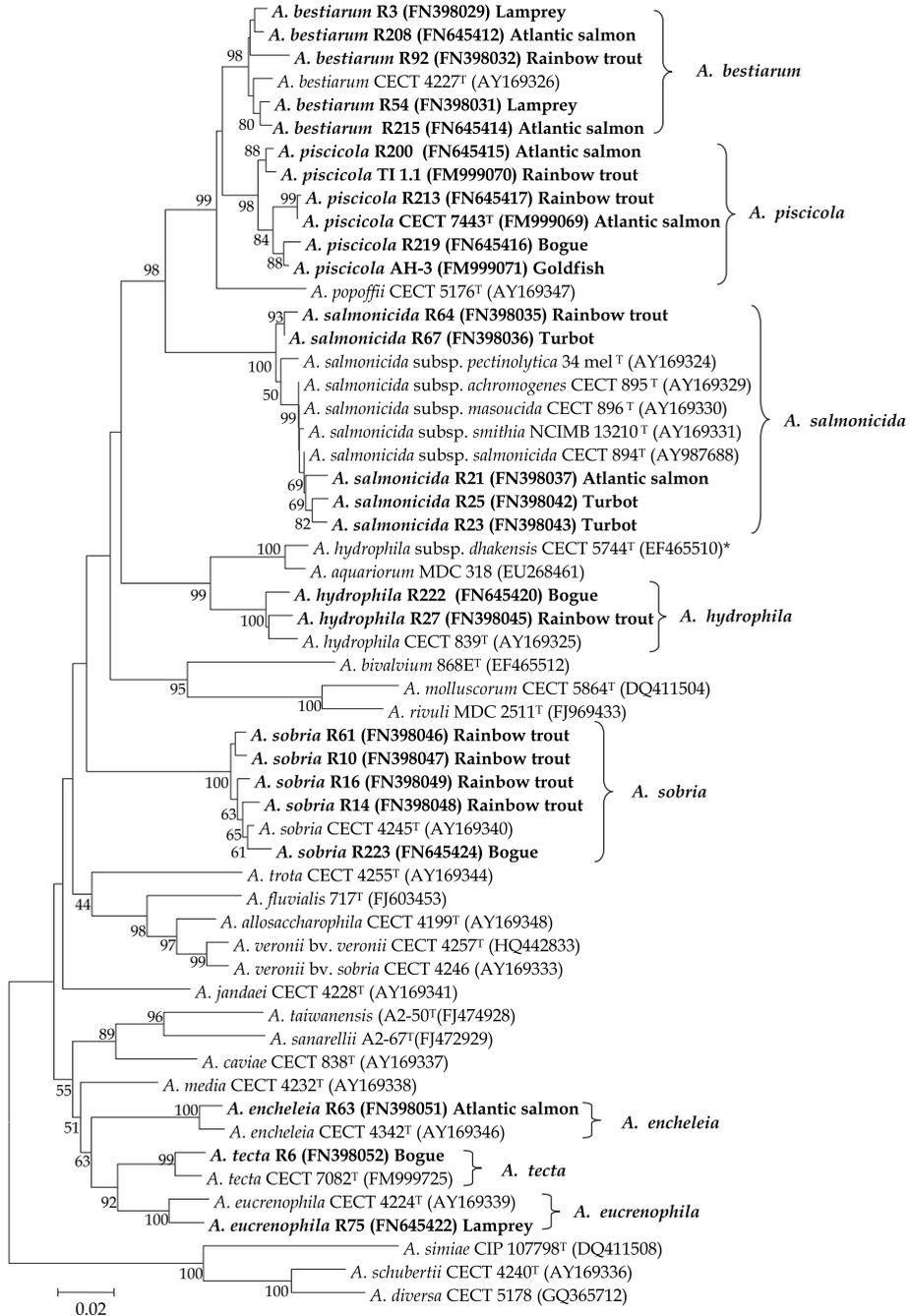


Fig. 1. Neighbor-joining phylogenetic tree based on *rpoD* gene sequences showing the relationships of 26 *Aeromonas* strains isolated from diverse diseased fish (in bold) (Beaz-

Hidalgo et al., 2010) with the 25 species included presently in the genus. The scientific names of the fish species: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), lamprey (*Petromyzum marinus*), bogue (*Boops boops*), goldfish (*Carasius auratus*), turbot (*Scophthalmus maximus*). **A. hydrophila* subsp. *dhakensis* is considered a synonym of the species *A. aquariorum* (Figueras et al., 2011b).

The first *Aeromonas* open access MLST scheme (<http://pubmlst.org/aeromonas>) was recently constructed (Martino et al., 2011) and another that included a set of 7 housekeeping genes (Lamy, 2011) was presented at the 10th International Symposium on *Aeromonas* and *Plesiomonas*. The open access MLST uses 6 genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA* and *recA*, 3,084 nt) and was applied to a total of 96 reference and field strains that included a high proportion of strains, 79.2% (76/96) obtained from known diseased freshwater and marine fish species mostly collected in the north-eastern area of Italy (Martino et al., 2011). The phylogenetic tree constructed with the concatenated sequences grouped the 76 fish strains, in order of prevalence, in *A. veronii* (n=28), *A. sobria* (n=25), *A. salmonicida* (n=6), *A. bestiarum* (n=6), *A. allosaccharophila* (n=4), *A. media* (n=4), *A. hydrophila* (n=2) and *A. encheleia* (n=1). The strains of the prevailing species *A. veronii* and *A. sobria* came from 11 and 6 fish species, respectively. However, the majority of the strains of *A. veronii* were from catfish (7 strains belonging to 7 different STs) followed by carp (6 strains belonging to 6 different STs) while *A. sobria* prevailed in trout (13 strains belonging to 12 different STs).

Recent studies have used the above-mentioned molecular methods (the 16S rDNA-RFLP and/or the housekeeping genes *rpoD* or *gyrB*) and have identified *A. salmonicida* subsp. *salmonicida* in farmed Arctic charr (*Salvelinus alpinus*) in Austria (Goldschmit-Clermont et al., 2009), *A. bestiarum* in farmed carp in Mexico (Soriano-Vargas et al., 2010), *A. sobria* from an outbreak that occurred in tilapias in China (Li & Cai, 2011), *A. veronii* from catfish in United States (Nawaz et al., 2006) and the studies carried out by Kozińska et al. (2002), Kozińska (2007) and Beaz-Hidalgo et al. (2010) that characterized numerous *Aeromonas* species isolated from diseased fish in Poland and in Spain respectively. Jun et al. (2010), using a multiplex PCR (m-PCR) and the 16S rRNA gene, identified *A. hydrophila* as the agent responsible for an outbreak that killed 50% of a farmed population of Korean cyprinid loach (*Misgurnus anguillicaudatus*). Nam & Joh (2007) using the 16S rDNA-RFLP found that 84% (252/300) of the isolates obtained from diseased trout belonged to the species *A. sobria*, and also found other *Aeromonas* species in a lower prevalence i.e. *A. encheleia* (9.3%), *A. salmonicida* (3.7%) and *A. bestiarum* (3%). Using several housekeeping genes (*gyrB*, *rpoD*, *dnaJ*, *recA*) also enabled Han et al. (2011) to identify *A. salmonicida* from ulcer lesions and haemorrhages in the black rockfish (*Sebastes schlegeli*) in Korea. These authors also sequenced the *vapA* gene (encoding the A-protein, a subunit of the A-layer or S-layer) and found that the sequences from the isolated *A. salmonicida* strains clustered with *A. salmonicida* subsp. *masoucida* in a neighbour-joining phylogenetic tree that included the sequences of other atypical *A. salmonicida* strains isolated from fish and of the subspecies *salmonicida*, *masoucida* and *smithia* (Han et al., 2011). However, these and other authors (Lund & Mikkelsen, 2004) stated that many atypical strains do not cluster with any of the subspecies of *A. salmonicida* when using the *vapA* sequences, and consider this gene not to be useful in the delineation of *A. salmonicida* to the subspecies level.

Pridgeon et al. (2011) sequenced the 16S-23S rDNA intergenic spacer region and 3 housekeeping genes (*cpn60*, *gyrB* and *rpoD*), to identify 6 isolates as *A. hydrophila* from

catfish (of which 3 were associated to an outbreak) and used suppression subtractive hybridization to investigate genome differences between a highly virulent and a less virulent strain, and they found 64 different sequences. By performing specific PCR reactions targeting these sequences, they determined that 3 of them that encoded a hypothetical protein XAUC_13870, a putative methyltransferase and a structural toxin protein RtxA were specific for the virulent strain. The specifically designed PCRs for these 3 sequences were further tested in another 7 field isolates (6 of them obtained from fish) for which the virulence had previously been determined by *in vivo* experiments in catfish. The two sequences encoding the hypothetical protein XAUC_13870 and the putative methyltransferase were present in the 4 highly virulent *A. hydrophila* strains but absent in the 5 less virulent strains, suggesting that these two sequences might be new virulence factors and therefore useful molecular markers for identifying highly virulent isolates of *A. hydrophila*.

4.2.2 PCR methods for the detection of *A. salmonicida* from fish tissue and water

Molecular PCR methods are fast, sensible and specific and have therefore been developed and applied for detecting typical and atypical *A. salmonicida* strains producing fish diseases (Tables 1-5). The methods that rely on the 16S rRNA gene have not been taken into consideration, because as commented above this gene does not enable the correct separation of *A. salmonicida* from its closely related species *A. bestiarum* and *A. piscicola* because they have an almost identical 16S rRNA gene sequence.

Table 1 describes in chronological order the most relevant PCR methods. In 1992, Gustafson et al. and Hiney et al. designed primers targeting 2 specific regions of *A. salmonicida*, which were later used by several authors for detecting the pathogen in water and fish samples (Tables 1, 2). The method described by Gustafson et al. (1992) targeted the *vapA* gene (which encodes the A-protein of the A-layer, as commented previously) of *A. salmonicida*. The authors validated their PCR method with 54 *A. salmonicida* strains (28 typical and 26 atypical) and found only one negative strain that was also not able to express the protein forming the A-layer. The authors suggested that the *vapA* gene in this strain possessed a mutation that affected both the amplification and the expression of the protein. However, they also found that 13 PCR positive strains did not express the protein (negative by Western blot) indicating that in those strains the mutations in the sequence of the *vapA* gene affected only the expression but not the PCR amplification. Further PCR analysis with primers covering the whole gene revealed that strains not expressing the A-layer had amplicons of lower molecular weight compared to strains expressing the protein, indicating that the mutations were deletions. The sensitivity of the PCR method was also validated using artificially infected samples that were prepared by inoculating a strain of *A. salmonicida* in the fish tank water and into homogenates of fish tissue and faeces of rainbow trout. The sensitivity from the direct detection in fish tissue was of 10 CFU/mg while in pure culture suspensions and water it was 1 CFU/ml (Table 1). The method was further tested using fish tissue and faecal samples of 25 naturally infected dead fish and of 25 suspected carrier fish, evaluating in parallel the results of the PCR carried out both before and after an enrichment incubation step in nutrient broth. Results for all 25 infected dead fish were positive with both methodologies (with and without a pre-enrichment). However, 5 of the 25 carrier fish were only positive after the pre-enrichment. Furthermore, the 10 water samples taken from

Reference	Target region or gene (bp)	N° of <i>As</i> strains evaluated	Type of samples	Results and/or conclusions
Gustafson et al. (1992)	<i>vapA</i> (421 bp) coding for the A protein (A or S layer).	28 typical (<i>Ass</i>), and 26 atypical <i>As</i> .	Dilutions of pure culture. Experimentally inoculated kidney, spleen and faeces homogenates and samples of sterile water. 25 dead infected fish, 25 suspected carrier fish and their tank water.	All typical <i>Ass</i> and atypical <i>As</i> strains were positive except 1 typical <i>Ass</i> strain. Detection limits were: 1 CFU in pure culture and in 100 ml of inoculated water and 10 CFU/mg in all tissue. All faecal and tissue samples of the 25 infected and 20 carriers fish were positive, 5 carrier fish were only positive after an enrichment step, therefore this step is necessary to eliminate false negatives.
Hiney et al. (1992)	DNA fragment (423-bp) recognized by Southern blot with unknown function ¹ .	25 <i>As</i> .	Dilutions of pure cultures.	All <i>As</i> were positive by dot blot hybridization with no cross-reaction with any of the 14 <i>Aeromonas</i> strains belonging to other 4 different species. Detection limit for the PCR assay from pure cultures was 2.4 cells.
Miyata et al. (1996)	DNA RAPD fragment (512 bp) of unknown function ² .	10 <i>Ass</i> , 2 <i>Asa</i> , 1 <i>Asm</i> and 4 atypical <i>As</i> .	Dilutions of pure cultures. Kidney of 15 experimentally infected fish.	The method detects only typical strains (the 10 <i>Ass</i> were positive) because the 2 <i>Asa</i> , the 1 <i>Asm</i> and the 4 atypical strains tested were negative ³ . The detection limit in pure cultures was 10 fg of bacterial DNA. A positive reaction was obtained from kidney samples from all the experimentally infected fish.
Høie et al. (1997)	DNA plasmid fragment of <i>Ass</i> and <i>Asa</i> of unknown function (710 bp).	2 <i>Ass</i> , 1 <i>Asa</i> and 1 <i>Asm</i> .	Dilutions of pure cultures of 4 strains of <i>Ass</i> , 1 of <i>Asm</i> and 1 of <i>Asa</i> . Experimentally inoculated kidney and gill homogenates. Kidney and gill from 109 covertly infected Atlantic salmon. Kidney from 88 wild brood Atlantic salmon.	All strains were positive except the only <i>Asm</i> tested. The PCR products were confirmed by hybridization. Detection limit in inoculated kidney and gill homogenates was 10 ⁴ CFU/100 ml. Kidney and gill samples from covertly infected Atlantic salmon were negative by PCR and by culture. <i>Ass</i> was not detected in kidney of brood salmon by PCR but 6/88 were positive by culture. However, a PCR based on the 16S rRNA gene of <i>As</i> was positive for 29 of the 88 kidney samples analyzed ⁴ .

Table 1. Developed PCR methods for the detection of *A. salmonicida* from diverse infected fish tissue and water.

Reference	Target region or gene (bp)	N° of <i>As</i> strains evaluated	Type of samples	Results and/or conclusions
Oakey et al. (1998)	5 RAPD fragments of unknown function were used as probes in Southern blot experiments.	10 <i>Ass</i> , 6 <i>Asa</i> , 4 <i>Asm</i> , 1 <i>Ass</i>	Dilutions of pure cultures.	All 5 Southern blot probes hybridize with <i>Ass</i> and with some of the subspecies, therefore the method does not discriminate typical from atypical <i>As</i> strains.
Nilsson et al. (2006)	DNA fragment (749 pb) of the insertion sequence (ISasa4) of the <i>tapA</i> gene encoding a protein of the type IV pili.	29 atypical <i>As</i> , 24 typical (<i>Ass</i>)	Dilutions of pure cultures.	This PCR differentiates most of the atypical <i>As</i> (27/29 were positive) from typical <i>As</i> (24/24 were negative). The detection limit was approx. 250 fg of template.
Beaz-Hidalgo et al. (2008)	<i>fstA</i> (422 bp) encoding a ferric siderophore receptor.	66 <i>Ass</i> , 2 <i>Asa</i> , 1 <i>Asm</i>	Dilutions of pure and mixed bacterial cultures. Experimentally inoculated (kidney and skin) tissue homogenates, mucus and blood. Mucus and blood of 31 wild salmon.	All <i>As</i> strains tested were positive, so it does not differentiate typical from atypical strains. Detection limits from pure cultures was 20-200 cells/ml and from mixed cultures 60-600 cells/ml. The latter was approx. the same detection limit for 100 mg of infected tissue with pure and mixed cultures. Detection limits from mucus was 10 ² cells/ml and in blood 10 ⁵ cells/ml. 6/31 samples of wild salmon were positive.
	<i>gyrB</i> (760 bp) encoding the B subunit of the DNA gyrase.		Dilutions of pure cultures.	The <i>gyrB</i> primers amplified all 69 <i>As</i> strains but showed less sensitivity when compared with the <i>fstA</i> primers, so no other experiments for <i>gyrB</i> were performed.

As, *A. salmonicida*; *Ass*, *A. salmonicida* subsp. *salmonicida*; *Asa*, *A. salmonicida* subsp. *achromogenes*; *Asm*, *A. salmonicida* subsp. *masoucida*; CFU, colony forming units.

¹A Blast analysis we carried out revealed that it shares 99% of similarity with the gene *mobA* (accession number: AJ508382) which is a mobilization protein of 1263 bp localized in the plasmid PAsa1 of *A. salmonicida* subsp. *salmonicida* (strain JF2267).²The sequence of this fragment is not available for comparison. ³This method has been used in several studies (Table 2) to differentiate the atypical strains based on the negative PCR reaction obtained with this method. ⁴We found that these 16S rRNA primers do not differentiate *A. salmonicida* from *A. bestiarum* and *A. piscicola*.

Table 1. Developed PCR methods for the detection of *A. salmonicida* from diverse infected fish tissue and water. (continued)

the tanks of the suspected carrier fish were only investigated using the enrichment step and all were positive. Gustafson et al. (1992) concluded that the enrichment step increases sensitivity and avoids possible false negative results (Table 1).

Hiney and co-workers (1992) discovered an apparently specific DNA fragment (423 bp) from a genomic DNA library of strain 7222V of *A. salmonicida* by differential hybridization with a strain of *A. hydrophila*. They tested the specificity of this fragment by dot blot hybridization using 25 *A. salmonicida* strains, other *Aeromonas* species (8 *A. hydrophila*, 3 *A. caviae*, 2 *A. sobria*, 1 *A. media*) and 11 strains belonging to other genera. The results demonstrated that the probe only reacted with *A. salmonicida*. This DNA fragment was isolated, sequenced and a specific PCR was designed obtaining a sensitivity of 2.4 cells of *A. salmonicida* in pure cultures (Table 1). Despite the authors sequenced the amplified fragment, they were not able, at that time, to determine the identity of this sequence. In the search for establishing its identity, now that the complete genome of *A. salmonicida* is known and that more sequences are available for comparison at the GenBank, we found that it shares 99% similarity with the sequence of the gene *mobA* (access number: AJ508382), which is a mobilization protein of 1263 bp localized in the plasmid PAsa1 of *A. salmonicida* (Fehr et al., 2006). The PCR method designed by Hiney et al. (1992) has been used and evaluated in posterior studies which are listed in Table 2.

In 1996, Miyata et al. sequenced a DNA fragment (512 bp) of unknown function obtained from a RAPD amplification of *A. salmonicida* and developed a PCR for the early detection of furunculosis, evaluating the method with several typical and atypical strains of *A. salmonicida* and experimentally infected fish. They found amplification only for the 10 *A. salmonicida* subsp. *salmonicida* strains (Table 1). The method could therefore be considered specific for detecting only typical furunculosis or typical *A. salmonicida* strains (i.e. those belonging only to the subsp. *salmonicida*). The assay was also positive in kidneys of 15 experimentally infected amago salmon (*Oncorhynchus rhodurus* subsp. *macrostomus*). So far, the sequence of this fragment has not been deposited in the GenBank and since this target has also been used in several later studies (Table 2), it is important to obtain the sequence in order to know its function.

Other studies performed by Høie et al. (1997) and Oakey et al. (1998) also developed PCRs for targeting DNA fragments of *A. salmonicida* of unknown functions, but these methods were not able to differentiate the subspecies. To our knowledge, these protocols have not been used since in any later studies and the main results of the two studies are summarised in Table 1.

Two new PCR methods were described in the first decade of the 21st century (Beaz-Hidalgo et al., 2008; Nilsson et al., 2006). The one of Nilsson and co-workers (2006) is based on the presence of an insertion element (ISE) in the *tapA* gene (encoding a pili subunit protein of the type IV pili) of *A. salmonicida*, which they named ISAsa4. They demonstrated that ISAsa4 was present in multiple copies (>30) but only in atypical strains of *A. salmonicida*, because none of the 24 typical strains tested were PCR positive, whereas 27/29 atypical strains were (Table 1). The identity of the 53 typical and atypical strains was confirmed by their positive amplification using the PCR method described by Gustafson et al. (1992) (which detects both typical and atypical strains) and by the non-amplification obtained for the 29 atypical strains using the method described by Miyata et al. (1996) (which only detects typical strains i.e. *A. salmonicida* subsp. *salmonicida*). On the basis of their results, Nilsson and co-workers

(2006) proposed this method as useful for differentiating most atypical strains. Furthermore, by Southern blot, using the ISasa4 as a probe, and after sequencing the *tapA* gene, these authors also observed that the atypical strains showed a high heterogeneity in this region/gene in comparison with the typical strains. Similar results were obtained previously when the amino acid sequences of the *vapA* gene from typical and atypical strains were compared, because identical sequences were found in typical strains, which contrasted with the significant variability observed in atypical strains (Lund & Mikkelsen, 2004). We believe that this high variability could indicate that the group of atypical strains may in fact not only embrace other subspecies of *A. salmonicida* but also other misidentified *Aeromonas* species. This PCR method directed at atypical *A. salmonicida* strains was used recently by Godoy et al. (2010) for characterising strains isolated from freshwater Atlantic salmon (*Salmo salar*) with successful results (Table 2), which we will discuss later.

The method developed by Beaz-Hidalgo et al. (2008) targets a fragment of 422 bp of the *fstA* gene of *A. salmonicida* that encodes a siderophore receptor and was validated by testing 69 strains of this species using Gustafson's method in parallel. All of the 69 strains produced the expected amplicon of the *fstA* gene, although 4 strains did not amplify with the method designed by Gustafson et al. (1992). This is probably due to the presence of mutations in the targeted DNA sequence of the *vapA* gene as Gustafson et al. (1992) discussed. The detection limit of the *fstA* PCR in dilutions of pure and mixed cultures (*A. salmonicida*, *Vibrio anguillarum* and *A. hydrophila*) was within the range of 20 to 600 cells/ml. In artificially infected kidney and skin samples the range was relatively similar, 60-600 cells per 100mg of tissue (Table 1). The proposed PCR for *A. salmonicida* was considered a non-destructive diagnostic tool when used in blood or mucus. Detection limits of experimentally infected mucus and blood samples were 2.5×10^2 and 1.5×10^5 CFU/ml respectively. Bacteria present in seeded blood was only detected at a high concentration (1.5×10^5 CFU/ml), which is probably due to interferences with heparin or other unknown blood components that compete with the bacterial DNA in the PCR amplification (Beaz-Hidalgo et al., 2008). This problem was reported by other authors previously (Høie et al., 1997 and references therein) and could maybe be avoided including a pre-enrichment step (Byers et al., 2002b; Gustafson et al., 1992) or evaluating the quality of the extracted DNA for the presence of inhibitors (Mooney et al., 1995). However, none of these approaches were tested by Beaz-Hidalgo et al. (2008). In the search for possible asymptomatic carrier fish, the *fstA* protocol was further assayed in the mucus and blood of 31 wild salmon that showed no signs of furunculosis, obtaining 4 *A. salmonicida* PCR-positive samples of mucus and 6 of blood recovered from 6 (19%) of the 31 salmon assayed (Table 1). On the other hand, culture methods were only able to isolate *A. salmonicida* from one blood sample, indicating that the PCR method had a higher sensitivity. It was concluded that the method was fast, specific and sensitive to *A. salmonicida* in both infected and asymptomatic carrier fish (Beaz-Hidalgo et al., 2008). The possible application to mucus and blood, which can be obtained without the need to perform necropsies or to sacrifice the fish, was a differential and advantageous characteristic over other molecular methods that were designed to detect the pathogen only from internal organs. Beaz-Hidalgo et al. (2008) within the same study also designed an additional method that relied on the amplification of the *gyrB* housekeeping gene of *A. salmonicida* and that produced the expected amplicon for the 69 strains tested. However, no further experiments were carried out with the *gyrB* PCR because its detection limit was higher than that obtained for the *fstA* gene (Table 1). Both the *fstA* and *gyrB* primers designed by Beaz-

Hidalgo et al. (2008) have been employed in later studies (Table 3) and have also been used in m-PCR methods developed for the simultaneous detection of *A. salmonicida* and other bacterial species that cause fish diseases (Table 4, section 4.2.5). In our laboratory, we have recently evaluated 10 strains of each of the species *A. piscicola*, *A. bestiarum* and *A. salmonicida* (including the type strains of all the *A. salmonicida* subspecies) using the PCR method described by Beaz-Hidalgo et al. (2008), which targets a fragment of 422 bp of the *A. salmonicida* *fstA* gene. This assay was carried out to know whether the primers only reacted with *A. salmonicida* or could also react with the closely-related species *A. piscicola* and *A. bestiarum*. To our knowledge those primers have never been tested in the recently described species *A. piscicola* (Beaz-Hidalgo et al., 2009) and only in the type strain of *A. bestiarum* (Beaz-Hidalgo et al., 2008). The results confirmed the specificity of the method for *A. salmonicida* because only the 10 strains of this species showed a unique band of expected size of the *fstA* gene. In contraposition none of the *A. piscicola* and *A. bestiarum* strains showed this specific band (422 bp) despite some bands of others sizes were amplified in some strains (unpublished results).

Another potential new specific target of *A. salmonicida* is a hypothetical protein named AssHPA, which was discovered by chance by Kingombe et al. (2010) while they were developing a m-PCR for the detection of three enterotoxins (*act*, *alt* and *ast*). Since the AssHPA amplicon (148 bp) was present in the nine *A. salmonicida* strains isolated from dead fish, the authors suggested that its usefulness as a specific target for the identification of this species could be evaluated. However, it was also present in two other strains, one identified as *A. bestiarum* and one identified as belonging to the *A. caviae/A. media* phenotypic complex. Considering this, we believe that the specificity of this PCR reaction might be compromised by this cross reactivity with other *Aeromonas* species and so requires further investigation with a greater number of strains of other species, including *A. piscicola*. The latter should also be evaluated with all the molecular methods available that can differentiate *A. salmonicida* strains (typical and atypical). This would guarantee that *A. piscicola*, which is commonly infecting fish, would not interfere by producing false positive reactions.

4.2.3 Application or comparative evaluation of the described PCR methods

Several studies have evaluated individually, or compared, the methods described in Table 1 and the results and conclusions are summarized in Table 2. In this sense, Morgan et al. (1993) used the PCR method designed by Hiney et al. (1992) to confirm the presence of viable not culturable (VNC) cells of a strain of *A. salmonicida* after 21 days incubation in sterile and untreated lake water microcosms (Table 2). Flow cytometry was used to determine the number of viable cells comparing it with the amount recovered after cultivation on agar plates. In the sterile microcosm culturable *A. salmonicida* cells were evaluated on trypticase soy agar (TSA) plates. However, to differentiate this species from other indigenous bacteria present in untreated lake water, the strain tested was marked using a kanamycin resistant plasmid and the *xyIE* gene (that encodes a D-xylose transporter). Growth of colonies on TSA agar plates supplemented with kanamycin revealed the presence of the tested *A. salmonicida* strain in the untreated water. The bacteria were also detected using the PCR designed by Hiney et al. (1992), which targets the *vapA* gene and in the case of the untreated water also the specific PCR targeting the *xyIE* gene only present in the marked *A. salmonicida* cells. After 21 days, counts of viable cell measured by flow

cytometry were 4 logarithms higher than culturable cells in the sterile microcosm, and a positive PCR detection was obtained at all the times tested (Table 2). In the untreated microcosm, the number of culturable *A. salmonicida* cells had declined below the detection limit after 14 days, although both viable cells and positive PCR amplicons (confirmed by hybridization) were observed until the end of the experiments.

The same DNA fragment discovered by Hiney et al. (1992) was also used by O'Brien et al. (1994) to investigate the presence of *A. salmonicida* in samples of faeces, effluent and tank water of a hatchery of Atlantic salmon smolts. Their results showed that although cultures of water and particulate matter of effluents of tanks with apparently healthy fish were negative for *A. salmonicida*, the pathogen was detected by PCR. The estimated sensitivity was approximately 1000 fg of *A. salmonicida* DNA (or 200 genome equivalents) per g of sample by PCR detection, but this sensitivity increased 10 times by dot blot hybridization (Table 2).

Byers and colleagues (2002a) compared three PCR methods designed by Gustafson et al. (1992), Hiney et al. (1992) and Miyata et al. (1996) in order to verify the identity of 308 *A. salmonicida* strains, which included type and reference collection strains as well as strains recovered from 38 teleost fish. They determined their sensitivity in inoculated fish tissue homogenates and evaluated the method on mucus and other samples (gill, muscle lesion, intestine, spleen and kidney) from experimentally infected salmonids (Table 2). They found that the methods of Hiney et al. (1992) and Gustafson et al. (1992) correctly identified 92.5% and 93.5% of the 308 strains (typical and atypical) and their simultaneous use gave a positive result for 99.4% of the strains (Table 2). In agreement with what it was described by Miyata et al. (1996) their PCR was able to identify 100% of the *A. salmonicida* subsp. *salmonicida* strains but none of the atypical strains. The latter were positive only with the methods of Hiney et al. (1992) and Gustafson et al. (1992). Thus they concluded that the simultaneous application of these three methods appeared to be useful for distinguishing typical and atypical isolates of *A. salmonicida*. Byers et al., (2002a) found that some PCR negative isolates for the method of Hiney et al. (1992) targeting the *vapA* gene of the A-layer, were still able to produce this layer and suggested that this was probably due to a mutation within the primer site that did not affect the expression of the gene as it was already described in the original study of Hiney and co-workers. They found that the methods of Hiney et al. (1992) and Gustafson et al. (1992) were more sensitive than that of Miyata et al. (1996) (Table 2). The obtained sensitivity in pure cultures ranged from 0.2 to 2 pg of DNA in 50 μ l, while in the different inoculated fish tissues it ranged from 10^3 to 10^5 CFU per g. Furthermore, they detected the presence of the pathogen in tissue of all the experimentally infected fish (Byers et al., 2002a). In a second complementary study, Byers et al. (2002b) investigated the presence of *A. salmonicida* in covertly infected rainbow trout, Atlantic salmon and Arctic charr again using the same 3 PCR methods in parallel with conventional cultures. However, they only evaluated the two most sensitive methods (Gustafson et al., 1992; Hiney et al., 1992) for the direct PCR detection of *A. salmonicida* in experimentally infected fish. They concluded that culturing was a more reliable method than the PCR assays, and estimated that the lowest detection limit of the 2 PCR methods assayed (Gustafson et al., 1992; Hiney et al., 1992) was 4×10^5 CFU/g of tissue. In addition, for evaluating the presence of *A. salmonicida* in covertly infected fish, they also recommended the use of a pre-enrichment culture step of the tissue before the PCR assay (Byers et al. 2002b). Another interesting result was that mucus samples yielded PCR products more often

Reference	Objective	PCR method/name of primers	Type of samples	Results and/or conclusion
Morgan et al. (1993)	To study the viable but not culturable state (VNC) of <i>As.</i>	Hiney et al. (1992)/ AP	Experimentally inoculated sterile and untreated lake freshwater.	The number of bacteria recovered from inoculated sterile lake water after 21 days by conventional culture was only 4 CFU/100 ml. However 5.6×10^4 cells/ml viable cells were confirmed by flow cytometry. In untreated lake water colonies were not obtained after 14 days. However, the bacteria were detected over the 21-day experiment by PCR. <i>A. salmonicida</i> possess a VNC state in inoculated freshwater.
O'Brien et al. (1994)	To detect <i>A. salmonicida</i> in a hatchery of Atlantic salmon.	Hiney et al. (1992)/ AP	Faeces, effluent and tank water samples from a hatchery of Atlantic salmon smolts.	The method enables detection of <i>A. salmonicida</i> from all samples. Parallel culture techniques were negative in the effluent and tank water samples. The estimated detection limit of the PCR products by gel electrophoresis was approx. 200 <i>A. salmonicida</i> genome equivalents/g of sample but dot blot hybridization was 10 times more sensitive.
Byers et al. (2002a)	To compare the results obtained for the detection and identification of 308 <i>A. salmonicida</i> strains using three existing PCR methods in parallel.	Gustafson et al. (1992)/PAAS Hiney et al. (1992)/ AP Miyata et al. (1996)/MIY	Dilutions of pure cultures. Experimentally inoculated tissue homogenates (mucus, gill, kidney and intestine). Mucus, gill, muscle lesion, intestine, spleen and kidney from experimentally infected fish.	Of the 308 <i>A. salmonicida</i> typical and atypical strains tested the AP amplified 285 and the PAAS 288. Their parallel application improved the individual results and detected 306 (99.4%) strains. The MIY method only amplified the typical <i>Ass</i> strains. The AP and PAAS methods provided better sensitivity in pure cultures (0.2 pg-2 pg/50 µl) and in tissue (10^3 - 10^5 CFU/g) than the MIY method (200 pg-20000 pg/50 µl in culture and 10^6 - 10^7 CFU/g in tissue). Tissues from all the experimentally infected fish were positive.

Table 2. Studies that apply the PCR methods described in Table 1.

Reference	Objective	PCR method/name of primers	Type of samples	Results and/or conclusion
Byers et al. (2002b)	To compare the results obtained for the detection and identification of <i>A. salmonicida</i> in experimentally and covertly infected rainbow trout, Atlantic salmon and Artic charr using three existing PCR methods in parallel.	Gustafson et al. (1992)/PAAS Hiney et al. (1992)/AP Miyata et al. (1996)/MIY	Tissue (mucus, gill, spleen, kidney, intestine) of experimentally infected salmonids as well as covertly infected (or carriers) salmonids.	All experimentally infected fish were positive with AP and PAAS methods. The 3 PCR methods identified correctly the 32 isolates recovered from covertly infected fish by culture. Detection limit in covertly infected salmonids with the AP and PAAS methods was 10 ⁵ CFU/g. An enrichment step is required to increase the sensitivity.
Skugor et al. (2009)	To detect and determine levels of <i>A. salmonicida</i> in experimentally infected vaccinated and unvaccinated Atlantic salmon.	Hiney et al. (1992)/AP	Liver and spleen from experimentally infected vaccinated and unvaccinated Atlantic salmon.	<i>A. salmonicida</i> was detected in the liver and spleen of all analyzed fish but the load was substantially lower in vaccinated compared to unvaccinated fish.
Godoy et al. (2010)	To identify by PCR 5 biochemically characterized <i>A. salmonicida</i> (typical or atypical) strains isolated from a furunculosis outbreak in freshwater farmed Atlantic salmon.	Gustafson et al. (1992)/PAAS Miyata et al. (1996)/MIY Nilsson et al. (2006)/ISasa4	External lesions, kidney, liver, spleen, heart of moribund Atlantic salmon.	Since the 5 strains only amplified with the PAAS primers (that does not discriminate between typical and atypical) and with those described by Nilsson et al. (2006) (specific for atypical strains) but did not with the MIY primers (specific for typical strains), the strains were considered as atypical <i>A. salmonicida</i> .

Table 2. Studies that apply the PCR methods described in Table 1. (continued)

than tissues like the intestine (Byers et al. 2002b). The authors suggested that this might indicate either that the pathogen is more abundant in certain body parts or that some types of infected tissue may inhibit the PCR more than others. In our view, it should be considered that the mucus that covers all the surface of the fish is the most accessible part of the body to *A. salmonicida*, which could be another reason for these results.

Another study in which several molecular methods were used in parallel was the one performed by Godoy et al. (2010) that aimed at re-identifying 5 biochemically characterised *A. salmonicida* strains isolated from a furunculosis outbreak that occurred in Chile in a freshwater farm of Atlantic salmon. The study tried to determine if the infection was produced by typical or atypical *A. salmonicida* strains, since atypical furunculosis had been detected previously in seawater in Chile. They used the primers designed by Gustafson et al. (1992), which recognize both typical and atypical *A. salmonicida* strains, those of Nilsson et al. (2006), which targeted the repeated insertion element ISasa4 specific only of the atypical strains (non *salmonicida* subspecies), and those designed by Miyata et al. (1996), which only recognize typical strains (*A. salmonicida* subsps. *salmonicida*). The five isolates were positive for the PCR that targeted the insertion element ISasa4 (Nilsson et al., 2006) and the PCR that targeted the DNA region of Gustafson et al. (1992), but were negative for that of Miyata et al. (1996). These results indicated that the freshwater salmon isolates were atypical strains of *A. salmonicida*. Sequencing nearly the complete 16S rRNA gene was also carried out and revealed that the five isolates obtained from diseased Atlantic salmon reared in freshwater shared 100% similarity with *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida*, whereas with *A. salmonicida* subsps. *salmonicida* the similarity was 99.85%. In our view Godoy et al. (2010) forgot to consider that the species *A. bestiarum* shows an identical 16S rRNA gene sequence to *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida* (Martinez-Murcia et al., 2005). Furthermore, some strains of the recently proposed new species *A. piscicola* also share a 100% 16S rRNA gene similarity with strains of *A. bestiarum* and *A. salmonicida*, including the subspecies *achromogenes* and *masoucida*. When the authors tested another 12 atypical *A. salmonicida* strains isolated from cultured marine fish, they found that the ISasa4 PCR was negative. Godoy et al. (2010) suggested that this might be due to a lower number of copies of this insertion element being present in the atypical *A. salmonicida* strains they isolated from the marine environment, in comparison with those isolated from freshwater, or due to variations in the targeted sequence. However, none of these suggestions have been investigated experimentally or proven by sequencing the specific region.

4.2.4 Studies that develop other PCR technologies using previously described targets

Other authors have used the PCR primers or a part of the specific target region described in previous studies for developing other PCR technologies, such as a nested PCR (Mooney et al., 1995), quantitative PCR methods (Balcázar et al., 2007; Goodwin & Merry, 2009) or a loop mediated isothermal amplification (LAMP) (Kulkarni et al., 2009), which are all summarized in Table 3 and described in this section. In the study carried out by Mooney et al. (1995), they applied the method described by Hiney et al. (1992) for the detection of *A. salmonicida* in the blood of 61 wild Atlantic salmon, but all samples were negative. In order to verify this, the authors developed an improved extraction procedure and a test to evaluate the quality of DNA by performing a PCR suitability test. The latter consisted of a PCR targeting a region of the fish host genome in order to determine if the sample was free

of PCR inhibitors that might interfere with the PCR detection of *A. salmonicida*. Furthermore they developed a nested PCR procedure (involving two consecutive PCRs) to improve sensitivity. So, after the conventional PCR (using the primers of Hiney et al., 1992) was carried out, specific primers that further amplified a sub-region (278 bp) of the original amplicon (423 bp) were designed and used in a second PCR. In this way, Mooney et al. (1995) were able to obtain amplification bands at the expected size in 87% of the 61 fish blood samples (Table 3).

Balcázar et al. (2007) developed a quantitative real-time PCR (Q-PCR) for the detection of *A. salmonicida* in inoculated fish tissue and in naturally infected fish. The Q-PCR targeted 131 bp situated within the 423 bp of the *mobA* gene that Hiney et al. (1992) amplified with the advantage of being able to quantify the PCR product (Tables 1, 3). The specificity of the method was confirmed by the positive results obtained for the 16 *A. salmonicida* isolates tested (belonging to 3 subspecies: *salmonicida*, *achromogenes* and *masoucida*) and the negative results for 10 strains belonging to other *Aeromonas* species and for 16 strains of different bacterial genera. Sensitivity of the Q-PCR was similar in pure culture and in inoculated tissue and was within the range of 0.5 pg to 50 ng of DNA that was established to be equivalent to 16 CFU (Table 3). In order to further validate the designed Q-PCR, it was applied to fish from natural outbreaks of furunculosis, and the levels of *A. salmonicida* per g of tissue (kidney, liver and spleen) obtained ranged from of 5.12×10^2 to 1.05×10^4 CFU.

In a more recent study, Goodwin & Merry (2009) used the three pairs of primers previously described by Gustafson et al. (1992), Hiney et al. (1992) and Miyata et al. (1996) and adapted them for a Q-PCR to analyse 62 ulcer swab samples obtained from carp to investigate the possible association of typical and atypical *A. salmonicida* in the ulcerative disease of the carp. Using the conventional PCR, they demonstrated that atypical strains were present in 84% (52/62) of the carp ulcers analysed. Like in other studies (Byers et al., 2002a) the distinction between typical and atypical strains was based on the positive amplification obtained using the primers of Hiney et al. (1992) and Gustafson et al. (1992) and the negative amplification using those of Miyata et al. (1996). Sixteen percent (10/62) of the samples were found to be PCR negative, which coincided with the ulcer swab samples taken from carp that live in water with high temperatures. These authors suggested that these increasing temperatures are likely to negatively affect the survival of the pathogen or to help the carp immune system to eliminate it (Goodwin & Merry 2009). Using Hiney's primers, Goodwin & Merry (2009) calculated a range of 9.83×10^2 - 7.74×10^7 genome copies of *A. salmonicida* per μg of host DNA extracted from the ulcer swab samples of the carp (Table 3). In parallel, the authors also grew cultures of the swabs but failed to isolate the bacteria and they argued that this is most likely due the overgrowth of other bacteria, particularly fast-growing motile aeromonads (which might also be responsible for the disease) as occurs with gills and skin where dominant bacteria mask the presence of lower prevalence bacteria. The authors concluded that the Q-PCR protocol might be a useful new tool to study the *A. salmonicida* epidemiology of the carp ulcer (Goodwin & Merry 2009).

Another totally different method is the loop-mediated isothermal amplification (LAMP) assay developed by Kulkarni et al. (2009) for the detection of furunculosis in Atlantic cod (*Gadus morhua*). For the development of the method, the authors designed 5 sets of primers targeting the *gyrB* gene region described by Beaz-Hidalgo et al. (2008) as useful for detecting *A. salmonicida*. The LAMP technique was first described by Notomi et al. (2000) to detect the

Reference	Objective	Type of samples	Results and/or conclusion
Mooney et al. (1995)	To increase the sensitivity of the PCR of Hiney et al. (1992) improving the DNA extraction (developing a procedure for testing its quality) and performing a nested-PCR.	Blood samples from 61 wild Atlantic salmon.	Conventional PCR did not detect <i>A. salmonicida</i> in the blood of wild salmon but the improved method was positive for 53 of the 61 evaluated fish. These results were confirmed by hybridization. Sensitivity was < 100 <i>A. salmonicida</i> genome equivalents/fish sample.
Balcázar et al. (2007)	To develop a quantitative real time-PCR (Q-PCR) for the detection of <i>A. salmonicida</i> from fish tissue using a smaller fragment (131 bp) of the region targeted by Hiney et al. (1992).	Tissue of naturally infected fish recovered from outbreaks. Dilutions of pure cultures. Experimentally inoculated fish tissue homogenates (liver, kidney, spleen and intestine).	<i>A. salmonicida</i> was detected and quantified in all naturally infected fish (in concentrations ranging from 5.12×10^2 CFU/g to 1.05×10^4 CFU/g). Detection limits in pure cultures and in inoculated tissue (liver, kidney, intestine, spleen) was approximately 16 CFU per Q-PCR reaction.
Goodwin & Merry (2009)	To establish the incidence of typical and atypical <i>A. salmonicida</i> in ulcerative lesions of the carp using primers designed by Gustafson et al. (1992), Hiney et al. (1992) and Miyata et al. (1996) and to used them in a Q-PCR.	Swabs from ulcerative lesions of diseased carp.	52 of the 62 strains recovered from ulcers were considered atypical and therefore those are the As strains associated to the carp ulcerative disease. The range of genome of copies of <i>A. salmonicida</i> in ulcer swab samples was 9.83×10^2 to 7.74×10^7 / μ g of DNA.
Kulkarni et al. (2009)	To develop a LAMP protocol for the rapid, sensitive and specific detection of furunculosis in Atlantic cod using 5 <i>A. salmonicida</i> specific primers for the <i>gyrB</i> gene targeted by Beaz-Hidalgo et al. (2008).	Spleen obtained from experimentally infected and uninfected fish. Dilutions of pure cultures. Experimentally inoculated mucus from a healthy fish.	The LAMP assay was specific for <i>A. salmonicida</i> and was positive for all the experimentally infected fish samples. LAMP was more sensitive than conventional PCR detection in pure cultures (1 pg of DNA/ml vs. 100 pg of DNA/ml) and in infected mucus (10 pg of DNA/ml vs. 1000 pg of DNA/ml).

LAMP, loop-mediated isothermal amplification.

Table 3. Studies that improve the PCR methods described in Table 1 or use the target regions and/or primers to design other methods.

hepatitis B virus and has the advantages of being faster (45 min) than conventional PCR and of not needing a thermocycler or any sophisticated equipment. Furthermore, on-site detection of the pathogen can be carried out by direct visualization of the fluorescent staining (SYBR Safe green) of LAMP products under a handheld UV light (Kulkarni et al., 2009). In their work, Kulkarni et al. (2009) compared the LAMP assay with the conventional PCR using the *gyrB* and *fstA* primers previously designed by Beaz-Hidalgo et al. (2008). They tested serial DNA dilutions of a pure culture of *A. salmonicida* and achieved a sensitivity of 1 pg of bacterial DNA/ml with LAMP, which is a 100-fold improvement over conventional PCR detection. Both LAMP and conventional PCR were assayed inoculating mucus samples, and the detection limits of 10 pg of bacterial DNA/ml and 1000 pg of bacterial DNA/ml, respectively, were achieved (Table 3). The use of the LAMP technique has rarely been reported in the literature, the study by Kulkarni et al., (2009) being the first one to use it for the detection of an *Aeromonas* fish pathogen. Further validation studies are needed for it to be applied in diagnostic laboratories and in on-site programmes (Kulkarni et al., 2009).

4.2.5 Multiplex PCR (m-PCR) methods to detect simultaneously *A. salmonicida* and other fish pathogenic bacteria

The advantage of using an m-PCR is its ability to detect several targeted sequences simultaneously, enabling the presence of several pathogens to be evaluated at once, being less time consuming and more cost effective than any individual PCR. González et al. (2004) developed an m-PCR that could be adapted to a microarray that targeted *A. salmonicida* and 4 other important marine fish pathogenic bacteria i.e. *V. anguillarum*, *Photobacterium damsela* subsp. *damsela*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Table 4). For detecting *A. salmonicida*, 2 regions of the *vapA* gene encoding the A-layer were selected, a region of 177 bp different from that of Gustafson et al. (1992) and a smaller fragment (101 bp) of the same region (423 bp) described by Hiney et al. (1992). They tested a total of 75 strains, representing 28 species of several genera, including 3 strains of *A. salmonicida* subsp. *salmonicida* and 3 strains belonging to other *Aeromonas* species (Table 4). The specificity of the assay was 100%, but the authors indicated that false negatives might arise as result of naturally occurring mutations in the region targeted by the primers. The sensitivity obtained with pure bacterial cultures (4-5 CFU) was similar to that obtained with other previously discussed PCR methods (Altinok et al., 2008; Gustafson et al., 1992; Hiney et al., 1992; Onuk et al., 2010).

Altinok et al. (2008) described another m-PCR for the detection of *A. hydrophila*, *A. salmonicida* subsp. *salmonicida* and 3 other major bacterial fish pathogens (*Flavobacterium columnare*, *Renibacterium salmoninarum* and *Yersinia ruckeri*) (Table 4). For detecting *A. salmonicida*, a modification of the Hiney et al. (1992) primers were used, while for detecting *A. hydrophila* the primers were those previously described by Nielsen et al. (2001) targeting the 16S rRNA gene. However, when we searched for any possible cross reactivity of *A. hydrophila* with sequences of other *Aeromonas* species, we found that complementary sequences to the primer regions were also present in the species *A. molluscorum* (only known from shellfish) and *A. encheleia* (originally isolated from European eels in Valencia, Spain and found in association with fish diseases in other studies). This seems to indicate that those primers cannot be considered specific for detecting *A. hydrophila*, and this needs to be tested experimentally using several strains of the mentioned interfering species. The method

Reference	Simultaneous detection of:	Target region or gene (bp)	N° of <i>Aeromonas</i> strains and type of samples evaluated	Results and/or conclusion
González et al. (2004)	<i>A. salmonicida</i> <i>Photobacterium damsela</i> subsp. <i>damselae</i> , <i>Vibrio vulnificus</i> <i>Vibrio anguillarum</i> <i>Vibrio parahaemolyticus</i>	A different region of 177 bp of the <i>vapA</i> gene targeted by Gustafson et al. (1992) and a smaller fragment (101 bp) of the region (423 bp) targeted by Hiney et al. (1992) ¹ .	3 <i>A. salmonicida</i> subsp. <i>salmonicida</i> , 1 <i>A. caviae</i> , 1 <i>A. hydrophila</i> and 1 <i>A. sobria</i> . Dilutions of pure cultures.	Detection limit for <i>A. salmonicida</i> in pure cultures was <20 fg of genomic DNA, which is equivalent to 4-5 CFU. The m-PCR products were used to develop a microarray and clear hybridization signals for both regions were obtained.
Altinok et al. (2008)	<i>A. salmonicida</i> subsp. <i>salmonicida</i> <i>A. hydrophila</i> <i>Flavobacterium columnare</i> <i>Renibacterium salmoninarum</i> <i>Yersinia ruckeri</i>	A slightly smaller fragment (416 bp) of the region (423 bp) targeted by Hiney et al. (1992) ¹ for <i>A. salmonicida</i> . The region of the 16S rRNA gene proposed by Nielsen et al. (2001) for <i>A. hydrophila</i> .	5 <i>A. salmonicida</i> subsp. <i>salmonicida</i> , 4 <i>A. hydrophila</i> and 2 <i>A. sobria</i> . Dilutions of pure cultures. Tissue (liver, spleen, kidney, gill, skin and fins) of diseased farmed rainbow trout.	Detection limit in pure cultures was 1 CFU. Of the 558 diseased rainbow trout obtained from 31 farms, 112 were positive for the detection of any of the 3 pathogens including 35 positive for <i>A. hydrophila</i> and 22 for <i>A. salmonicida</i> .
Onuk et al. (2010)	<i>A. salmonicida</i> subsp. <i>salmonicida</i> <i>Flavobacterium psychrophilum</i> <i>Yersinia ruckeri</i>	Identical region (<i>fstA</i> , 422 bp) as Beaz-Hidalgo et al. (2008).	9 <i>A. salmonicida</i> subsp. <i>salmonicida</i> . Dilutions of pure cultures. Experimentally inoculated homogenates of liver tissue obtained from a healthy salmon.	Detection limits were 30 CFU in pure cultures and 250 CFU in infected liver. All <i>A. salmonicida</i> subsp. <i>salmonicida</i> were positive.
Kulkarni et al. (2010)	<i>A. salmonicida</i> <i>Francisella piscicida</i> <i>V. anguillarum</i> (three major pathogens in farmed Atlantic cod).	Identical region (<i>gyrB</i> , 760 bp) as Beaz-Hidalgo et al. (2008).	1 <i>A. salmonicida</i> Dilutions of pure cultures.	Detection limit of 10µg-50 ng DNA/ml in pure cultures. The only reference strain of <i>A. salmonicida</i> tested was positive.

¹Hiney et al. (1992) indicated that this sequence had an unknown function. However, a Blast analysis we carried out revealed that it shares 99% of similarity with the gene *mobA* (accession number: AJ508382), which is a mobilization protein of 1263 bp localized in the plasmid PAsa1 of *A. salmonicida* subsp. *salmonicida* (strain JF2267).

Table 4. Studies that develop multiplex-PCR (m-PCR) to detect simultaneously *Aeromonas* spp. (mainly *A. salmonicida*) and other fish pathogens.

appeared to be specific for the 5 strains of *A. salmonicida* evaluated, with no cross-reaction with the other bacteria (Altinok et al., 2008). The detection limits in dilutions of pure culture were 2 CFU for *A. hydrophila* and 1 CFU for *A. salmonicida*, respectively, and the assay detected *A. hydrophila* in 35 fish and *A. salmonicida* in 22 fish of the 558 diseased rainbow trout analysed (Table 4).

In a very recent study, Onuk et al. (2010) has developed an m-PCR for the simultaneous detection of *A. salmonicida* and two other bacteria (*Flavobacterium psychrophilum* and *Y. ruckeri*) able to produce contagious infections in salmonids (Table 4). The detection limit was 30 CFU of *A. salmonicida* from culture suspensions and 250 CFU from inoculated homogenated liver tissue (Table 4). For the design of the m-PCR, they assayed in parallel the *vapA* primers of Hiney et al. (1992) and those of the *fstA* of Beaz-Hidalgo et al. (2008), showing that the latter had better specificity as none of the 9 strains analyzed showed any false negative reactions or non-specific amplifications. False negatives were, however, obtained for 2 of the 9 *A. salmonicida* isolates (2.2%) using the primers of Hiney et al. (1992). The failure of some *A. salmonicida* isolates to amplify with the latter primers agrees with results obtained by Byers et al. (2002a), who reported no amplification in 23 of the 308 isolates (7.5%) examined with these primers.

For monitoring the presence of *A. salmonicida*, *Francisella piscicida* and *V. anguillarum*, considered the three most important pathogens in cultured Atlantic cod, Kulkarni et al. (2010) developed an m-PCR. For detecting *A. salmonicida* they used the primers of the *gyrB* gene designed by Beaz-Hidalgo et al. (2008). The m-PCR was specific for the detection of the single strain of *A. salmonicida* tested with no cross-reaction with the other pathogens tested simultaneously and had a good detection limit when assayed in bacterial suspensions (Table 4).

5. Impact of climate change in *Aeromonas* infections

Several studies have reported that climate change can affect the aquaculture sector and its production, as it may increase the vulnerability of cultured fish to diseases due to an increase in water temperature and/or to a decrease in water quality (Alborali, 2006; Karvonen et al., 2010; Marcogliese, 2008; Marcos-López et al., 2010; Mohanty et al., 2010; Tam et al., 2011). It is well known that small changes in water temperature alter both the fish metabolism and physiology that may have consequences for their growth, fecundity or feeding behaviour (Alborali, 2006; Marcogliese, 2008; Mohanty et al., 2010). An increase in water temperature causes fish to suffer thermal stress, making them more susceptible to infections (by opportunistic pathogens such as *Aeromonas* spp.) and degrading their environmental habitat, lowering oxygen concentrations and altering the levels of nutrients (Alborali, 2006; Karvonen et al., 2010; Marcogliese, 2008; Marcos-López et al., 2010; Tam et al., 2011). However, it also has to be considered that at a higher water temperature many bacteria replicate at a higher rate and they might therefore be more abundant, favouring the spread of infectious diseases (Marcos-López et al., 2010). Motile opportunistic *Aeromonas* species have an optimal growth temperature under laboratory conditions of 25-30°C, and the ideal water temperature for *A. salmonicida* to survive is within the range 12.8°C to 21.1°C (Tam et al., 2011 and references therein). Classically, outbreaks of *Aeromonas* septicemia and furunculosis are linked to a rise in temperature, usually occurring during spring and summer (Tam et al., 2011). Increased temperature may also lengthen the transmission

Target region or gene (bp)	Primer set- Sequence of oligonucleotides	Conditions	Reference
<i>vapA</i> (421)	AP-1- 5'-GGC TGA TCT CTT CAT CCT CAC CC-3' AP-2- 5'-CAG AGT GAA ATC TAC CAG CGG TGC-3'	94°C 1s 57°C 25s 73°C 25s	x 35 Gustafson et al. (1992)
Unknown region ¹ (423)	PAAS-1- 5'-CGT TGG ATA TGG CTC TTC CT-3' PAAS-2- 5'-CTC AAA ACG GCT GCG TAC CA-3'	94°C 1min 55°C 1min 72°C 1min	x 30 Hiney et al. (1992)
Unknown region (512)	MIY-1- 5'-AGC CTC CAC GCG CTC ACA GC-3' MIY-2- 5'-AAG AGG CCC CAT AGT GTG GG-3'	94°C 30s 60°C 30s 72°C 1min 72°C 5min	x 30 Miyata et al. (1996)
IS sequence ISasa4 (749)	ISasa4F- 5'-CCT GCA CCG CCT CAT TTC TC-3' ISasa4R- 5'-GAA AAC CCA GTG ATC TGA GC-3'	94°C 2.5min 94°C 30s 67°C 30s 72°C 30s 72°C 9.5min	x 30 Nilsson et al. (2006)
<i>gyrB</i> (422)	Asg1- 5'-TGG CAT GGA ACA TTC CTC CT-3' Asg2- 5'-GTC GCC TGC TTT TTC CAG CA-3'	95°C 3min 95°C 30s 57°C 30s 72°C 1min 72°C 5min	x 40 Beaz-Hidalgo et al. (2008) ²
<i>fstA</i> (760)	Fer3- 5'-CGG TTT TGG CGC AGT GAC G-3' Fer4- 5'- AGG CGC TCG GGT TGG CTA TCT-3'	92°C 3min 92°C 1min 60°C 1min 72°C 1min 72°C 5min	x 30 Beaz-Hidalgo et al. (2008)

¹ Blast analysis we carried out revealed that it shares 99% of similarity with the gene *mobA* (accession number: AJ508382), which is a mobilization protein of 1263 bp localized in the plasmid PASa1 of *A. salmonicida* subsp. *salmonicida* (strain JF2267). ²Amplification conditions were not indicated in that study so those indicated were from Kulkarni et al. (2010). All these methods detected typical and atypical strains except Miyata et al. (1996) that detected only typical and Nilsson et al. (2006) that detected only atypical.

Table 5. Primers and conditions of the most commonly used PCR methods for the detection of typical and atypical *A. salmonicida* strains.

season, leading to a higher prevalence of the disease and to more widespread epidemics (Karvonen et al., 2010). There might also be ecological alterations between species or strains, resulting in the emergence of new pathogenic strains affecting a wider range of hosts (Karvonen et al., 2010; Marcos-López et al., 2010). Furthermore, bacteria might show a greater virulence, for instance *A. hydrophila* has shown a greater virulence in largemouth bass (*Micropterus salmoides*) at warmer temperatures because of either reduced resistance of the host or to an increased expression of virulence factors (Marcogliese, 2008 and references therein).

In a recent study, Tam et al. (2011) carried out a regional impact assessment of climate change in relation to furunculosis investigating fish populations in two lakes in Canada.

Lake water temperatures from 1963 to 2001 were used for the model to project future lake temperatures (2011-2100) considering different scenarios and calculating the vertical and surface water temperatures over different time scales. They recognized a significant rise in air temperature since 1963 and in the mid 1990s they detected the occurrence of furunculosis when the mean air temperature was 12.8°C and summer mean water temperature was 15°C. Between 2011 and 2100 they predict that the summer mean water temperatures will rise from 15-15.5°C to 16.5-17.4°C, conditions being within the range of the survival temperatures of *A. salmonicida* (12.8-21.1°C). Furthermore, they noticed that the estimated range of temperature between 16.5 and 17.4°C is relatively near to the 18°C at which virulence of *A. salmonicida* is better expressed (Daher et al., 2011). Tam et al., (2011) considered that the effects of climate change might also be transferred to other anthropogenic impacts, such as contamination that impacts on water quality. This is another factor that might favour the development of furunculosis.

In conclusion, we could say that there is a general agreement that as a consequence of global warming, water temperature will increase and as a result endemic diseases like furunculosis will become more prevalent and more difficult to control in immunodepressed fish populations (Marcos-López et al., 2010). In the long term, aquaculture must respond to climate change by minimizing discharges into water ecosystems, try to mitigate the negative impacts of climate change in water quality and avoid fish crowding to minimize disease transmission (Marcos-López et al., 2010).

6. Conclusions and perspectives

Classically the species of *Aeromonas* implicated in fish disease that have been considered important in ichthyopathology were *A. salmonicida* and *A. hydrophila*. However, recently this panorama of species has expanded with the discovery of new species like *A. piscicola* and *A. tecta*, which have been isolated mainly from diseased salmonids and turbot. These new species might have an important role in fish pathology that needs to be explored in the future. Other species such as *A. veronii* or *A. sobria* seem to have a specific role in the pathology of catfish and trout, respectively. All these species should have remained masked under *A. hydrophila* when only biochemical identification methods were applied. Therefore these methods should be avoided as they provide results that misrepresent the real prevalence and/or diversity of the species.

The species *A. salmonicida* includes a broad diversity of strains, some able and others unable to produce pigment or be motile under laboratory conditions. This heterogeneous behaviour has led to the introduction of the terms 'typical' and 'atypical' strains (subspecies different from *salmonicida*), which in our view is very confusing. However, strains of both groups are known to cause furunculosis and ulcerative diseases in a variety of fish hosts, in which they produce similar clinical characteristics. Furthermore, there has been evidence over the years that the identification of the strains of *A. salmonicida* as belonging to the different subspecies (other than *salmonicida*) is both phenotypically and genetically complex. Therefore, it is probably time to realize that the separation of this species into different subspecies does not fulfil the aim of helping to clarify the identity of the isolates on the basis of stable phenotypic and genetic characters, but just makes the situation more difficult and confusing. We believe that after genetic confirmation, it is probably better to avoid the use of the terms 'typical' and 'atypical' and to refer to the strains simply as *A. salmonicida*.

In ichthyopathology, correct identification is essential for determining the true etiology of the disease during outbreaks at aquaculture facilities and this is the basis for establishing adequate treatment and prevention programmes. Fast and reliable detection of *Aeromonas* is a key element to minimise the impact of the infection. Moreover, the continuous monitoring of *Aeromonas* in fish farms (both the water and the fish) is needed because these bacteria are autochthonous of the aquatic environment and can act as opportunistic pathogens.

Phenotypic methods are unreliable for identifying *Aeromonas* species. Therefore it is necessary to direct efforts towards the use of suitable and reliable molecular techniques.

There is a broad panorama of PCR-based methods developed to detect *A. salmonicida* in fish tissue (mucus, blood, and other tissues) that have shown to provide a good specificity and the tendency in the future will be to continue using m-PCR methods that will enable to screen several pathogens simultaneously. Sequencing the housekeeping genes (i.e. *rpoD* and *gyrB*) of the strains recovered from fish have proven to be useful for identifying the *Aeromonas* species and its routine use will revert on the clarification of the diversity of species involved in fish disease.

Also more knowledge will be gathered in the future from the complete genomes of both bacteria and the infected host fish. In the genus *Aeromonas* there is only one complete genome of the 3 available belonging to a strain recovered from diseased fish (trout) of the species *A. salmonicida* (strain A449). It can be expected that soon other ones will be available. These genomes can provide important information about the expressed genes in the host, in response to vaccination or infection that can be useful for selecting resistant fish populations in the future.

It is clear that one of the predicted effects of climate change will be an increase in the water temperature, a tendency that has already been observed and that can change the fish's immunocompetence and susceptibility to disease and affect fisheries and aquaculture. There is evidence to suggest that emergence, distribution and transmission of many pathogenic bacteria like *Aeromonas* will increase under the effect of global warming but other anthropogenic impacts such as those derived from water contamination might also be important drivers in exacerbating the problem. Few studies have tried to predict the impact of climate change on fish *Aeromonas* infections and therefore this needs to be further explored in order to search for timely corrective measures that can be implemented to counter the effects of global warming.

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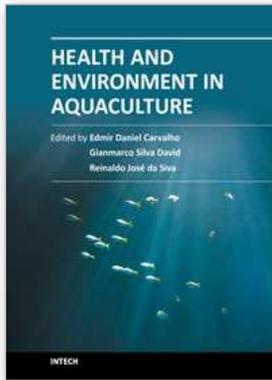
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Aquaculture has been expanding in a fast rate, and further development should rely on the assimilation of scientific knowledge of diverse areas such as molecular and cellular biology, and ecology. Understanding the relation between farmed species and their pathogens and parasites, and this relation to environment is a great challenge. Scientific community is involved in building a model for aquaculture that does not harm ecosystems and provides a reliable source of healthy seafood. This book features contributions from renowned international authors, presenting high quality scientific chapters addressing key issues for effective health management of cultured aquatic animals. Available for open internet access, this book is an effort to reach the broadest diffusion of knowledge useful for both academic and productive sector.

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