IPL
escola superior de turismo
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# *Development of a multiplex PCR tool for screening of pathogens in teleost farmed fish*

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# *Development of a multiplex PCR tool for screening of pathogens in teleost farmed fish*

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Title: Development of a multiplex PCR tool for screening of pathogens in teleost farmed fish

Título: Desenvolvimento de uma ferramenta de multiplex PCR para rastreio de agentes patogénicos em peixes teleósteos de aquacultura

*Faça as coisas o mais simples que puder, porém não se restrinja às mais simples.*

Albert Einstein

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## Resumo

A aquacultura desempenha um papel cada vez mais importante na produção de alimentos em todo o mundo (Martins *et al*., 2015). Espécies dos géneros *Aeromonas*, *Vibrio*, *Edwardsiella* e *Streptococcus* são patógenos que infetam peixes (Zhang *et al*., 2014), causando elevadas perdas económicas em aquacultura.

A corvina (*Argyrosomus regius,* Asso, 1801) é um dos maiores peixes da família Sciaenidae a nível mundial, e uma espécie de elevado valor comercial no Sudoeste da Europa, sendo neste momento objeto de grande interesse em todo o Mediterrâneo visando a sua produção comercial (Amoedo, 2011). Devido à sua elevada fertilidade, ampla distribuição e boa aceitação por parte dos consumidores torna-se um bom candidato à produção em aquacultura podendo atingir preços de mercado médio-altos (6 euros/kg, INE 2010).

Como ainda não foram reportadas manifestações patológicas relevantes nesta espécie, o principal método de prevenção é o controlo da densidade nos tanques, sendo necessário pesquisar eventuais patógenos que afetem a corvina em diferentes estádios do seu ciclo e mesmo em condições de stress.

O objetivo deste estudo foi o desenvolvimento de uma ferramenta de multiplex-PCR para a deteção precoce de *Edwardsiella tarda*, *Photobacterium damselae* subsp. *piscicida*, *Vibrio alginolyticus*, *Vibrio anguillarum* e *Vibrio harveyi* em peixes de aquacultura, incluindo a corvina, que permita de forma prática e eficiente a deteção destes patógenos.

Concluiu-se que é possível através de ferramentas de multiplex-PCR, a deteção dos patogénicos *Edwardsiella tarda*, *Vibrio alginolyticus*, *Vibrio anguillarum* e *Vibrio harveyi* até um limite mínimo de 0,4 ng de DNA/µl para *V. anguillarum*; 0.5 ng/µl para *E. tarda*; 1.5 ng/µl para *V. harveyi* e 5.6 ng/µl para *V. alginolyticus*, podendo, no futuro, estas técnicas ter aplicação prática cada vez mais extensa.

Palavras-chave: *Argyrosomus regius*, Aquacultura, diagnóstico de doenças, multiplex-PCR.

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## Abstract

Aquaculture plays an increasingly important role in food production worldwide (Martins *et al*., 2015). Species of the four genera *Aeromonas*, *Vibrio*, *Edwardsiella* and *Streptococcus* are major pathogens that infect fish (Zhang *et al*., 2014), causing high economic loss in aquaculture.

The meagre (*Argyrosomus regius*, Asso, 1801) is one of the largest fish from the Sciaenidae family worldwide, and a species of high commercial value in southwestern Europe. This species shows great interest for commercial production throughout the Mediterranean (Amoedo, 2011). Due to its high fertility, wide distribution and good acceptance by consumers, becomes a good candidate for aquaculture production that could reach medium-high market prices (6 euros/kg, INE 2010).

As have not yet been reported relevant pathological manifestations in this species, the primary method of prevention is to control the crop density, which requires research into possible pathogens affecting the meagre in different stages of the life cycle and even in stress conditions.

The aim of this study was to develop a multiplex-PCR tool for early detection of *Edwardsiella tarda*, *Photobacterium damselae* subsp. *piscicida*, *Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio harveyi* in farmed fish, including meagre, enabling practical and efficient detection of these pathogens.

In conclusion, it is possible through multiplex-PCR tools the detection of *Edwardsiella tarda*, *Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio harveyi* as low as 0.4 ng/µl for *V. anguillarum*; 0.5 ng/µl for *E. tarda*; 1.5 ng/µl for *V. harveyi* and 5.6 ng/µl for *V. alginolyticus*, may, in the future, practical application of these techniques increasingly extensive.

Keywords: *Argyrosomus regius*, Aquaculture, disease diagnostic, multiplex-PCR.

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### *Chapter II*. Development of a m-PCR tool for detection of four bacterial pathogens on Aquaculture

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FAO – Food and Agriculture Organization
HBSS – Hank’s Balanced Salt Solution
m-PCR – multiplex-PCR
NCBI – National Center for Biotechnology Information
PBS – Phosphate Buffered Saline
PCR – Polimerase Chain Reaction
Phdp – *Photobacterium damselae* subsp. *piscicida*
TSB – Tryptone Soya Broth

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# Chapter I.

# General Introduction

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## 1. Fish Diseases on Aquaculture

It may be assumed that fish are continually bathed in an aqueous suspension of microorganisms and most of the members of the normal microflora of water can be bacterial fish pathogen candidates (Chang *et al*., 2012). The appearance and development of a fish disease is the result of the interaction among pathogen, host and environment (Toranzo *et al*., 2005). Fish diseases, especially those caused by Gram-negative bacteria, are a serious problem in aquaculture (Castro *et al*., 2014). Regarding the infectious diseases caused by bacteria in marine fish, although pathogenic species have been described in the majority of the existing taxonomic groups, only a relatively small number are responsible of important economic losses in cultured fish worldwide (Toranzo *et al*., 2005). Disease outbreaks have direct effects on fish production, causing severe economic losses in the aquaculture sector (Martins *et al*., 2015). The possibility of outbreaks is increased if fish are stressed, as what happens with inappropriate water temperature, low dissolved oxygen, high nitrite levels, and high culture densities (Perera *et al*., 1997; Shoemaker *et al*., 2000). Healthy looking fish without any clinical signs or lesions can carry some pathogens and create a serious risk for spread of contagious diseases in fish populations (Onuk *et al*., 2010).

## 2. Traditional and Molecular diagnosis

Traditionally disease diagnosis is obtained by culturing bacteria on agar plates followed by phenotypic and serological characterization of the pathogen, or by histological examination (Pazos *et al*., 1996). Biochemical tests, DNA homology, and protease variability techniques have also been used (Chen *et al*., 1995), but these techniques have some disadvantages, such as the need for initial isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen, which can be overcome by molecular techniques such as polymerase chain reaction (PCR) used to increase sensitivity and specificity of pathogen detection (Altinok, 2011). Molecular methods have slowly established a place in the diagnosis of disease in aquaculture (Chatterjee and Haldar, 2012). Blood testing is preferable to tissue analyses because it does not require the sacrifice of the sampled fish and is suitable for monitoring fish on farms (Gonzalez *et al*., 2003).

## 3. Major bacterial pathogens on Aquaculture

In the last decade, edwardsiellosis, caused by *Edwardsiella tarda*, a Gram-negative, motile, rodshaped, member of the family Enterobacteriaceae has become an important bacterial pathogen in aquaculture (Castro *et al*., 2012). Affecting commercial fish species worldwide (Castro *et al*., 2011; Zhang *et al*., 2014) including flatfish, this bacteria also has been reported on environment contaminated by animals or man (Castro *et al*., 2014), being a possible source of zoonoses.

Members of the species *Photobacterium damselae* are frequently associated with disease outbreaks, and have been described as emergent fish pathogens in aquaculture systems (Martins *et al*., 2015). Photobacteriosis or Pasteurelosis is a septicemia caused by the halophilic, Gram-negative bacteria, *Photobacterium damselae* subsp. *piscicida*, a member of Vibrionaceae family which shares the same specie with *Photobacterium damselae* subsp. *damselae* (Osorio *et al*., 2000). It is commonly called "pseudotuberculosis" due to the fact that, in the chronic form, the diseased fish showed whitish tubercles in the internal organs which consist of bacterial colonies, necrotic phagocytes and granuloma in several internal organs of infected fish accumulations (Toranzo *et al*., 1991; Noya *et al*., 1995; Margariños *et al*., 1996). This disease is considered one of the most dangerous bacterial diseases in aquaculture worldwide due to its wide host range, high mortality rate, and ubiquitous distribution (Barnes *et al*., 2005; Andreoni and Magnani, 2014). The disease was first described in wild populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963, when a massive epizootic occurred in Chesapeake Bay (USA) (Snieszko *et al*., 1964). However, its taxonomic position remained controversial until DNA–DNA hybridization studies (Gauthier *et al*., 1995) provided evidence for its definitive reclassification in the genus *Photobacterium*, as *Photobacterium damselae* subspecies *piscicida* (Phdp) closely related to the subspecies *damselae* (Pdd) (Amagliani *et al*., 2009). Phenotypic homogeneity of *Photobacterium damselae* subsp. *piscicida* allows us to distinguish it from other subspecies - *Photobacterium damselae* subsp. *damselae* - by significant biochemical and physiological characteristics such as motility, nitrate reduction, the formation of gas from glucose and the production of hemolysin, urease, and amylase (Magariños *et al*., 1996; Romalde, 2002). The disease is important in Europe, where since 1990 there have been recorded several photobacteriosis outbreaks in different countries including Spain (Toranzo *et al*., 1991), France (Baudin-Laurencin *et al*., 1991), Italy (Ceschia *et al*., 1991), Greece (Bakopoulos *et al*., 1995) and Portugal (Baptista *et al*., 1996). Photobacteriosis seems to be more prevalent during the summer months (Frerichs and Roberts, 1989; Magariños *et al*., 1996) at higher water temperatures (greater than 23°C) and salinities (20-30) (Hawke *et al*., 1987; Magariños *et al*., 1996) and when water quality is low (Magariños *et al*., 1996). Although the optimum growth temperature of this microorganism is between 22.5°C and 30°C, Phdp can grow between 15°C and 32.5ºC (Magariños *et al*., 1992; Magariños *et al*.,1996). Moreover, it has been demonstrated that although Phdp cells may exist in a dormant state, they are capable of resuming rapid resuscitation and division when nutrient conditions are suitable (Magariños *et al*., 1994).

Vibriosis is one of the most important and the oldest recognized fish disease in marine aquaculture worldwide (Gonzalez *et al*., 2003). *Vibrio* spp. are Gram-negative and halophilic bacteria widely spread in sea- and brackish water worldwide (Messelhäusser *et al*., 2010). The main feature of this bacterial group is their capacity to cause serious alimentary intoxication associated with the consumption of raw or undercooked contaminated fish or shellfish posing a considerable public health threat as agents of sporadic and epidemic human infections, therefore representing an important microbial group in the field of food safety and quality (Espiñeira *et al*., 2010). Bacterial interaction or colonization with challenged organisms is a very complex process (Chatterjee and Haldar, 2012). During certain periods of the year, pathogenic *Vibrio* withstand unfavourable environmental conditions within aquaculture settings and when favourable environmental conditions are re-established, *Vibrio* are once again able to cause disease in wild animals (Chatterjee and Haldar, 2012). Ben-Haim *et al*. in 2003 advanced the hypothesis that aquaculture settings serve as foci or reservoirs for pathogenic *Vibrio* strains (Naylor *et al*., 2000; Chatterjee and Haldar, 2012). *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio ordalii* and *Vibrio vulnificus* are considered opportunistic pathogens of fish (Ghittino *et al*., 2003; Dalmasso *et al*., 2009; Zhang *et al*., 2014). Major *Vibrio* species *viz. V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus,* and *V. splendidus* are usually associated with shrimp diseases (Chatterjee and Haldar, 2012). *V. harveyi* is associated with luminescent vibriosis in shrimps e.g., *Litopenaeus vannamei* and *Penaeus monodon,* and it is the most important etiological agent for mass mortality in *P. monodon* (Lavilla-Pitogo *et al*., 1998; Lavilla-Pitogo and De la Pena, 1998; Austin *et al*., 2003; Guzmán *et al*., 2010; Chatterjee and Haldar, 2012). Internal symptoms of disease in fish caused by strains of *Vibrio* include intestinal necrosis, anaemia, ascitic fluid, petechial haemorrhages in the muscle wall, liquid in the air bladder, haemorrhaging and/or bloody exudate in the peritoneum, swollen intestine, haemorrhaging in or on internal organs, and pale mottled liver (Austin and Austin, 1999). External symptoms include sluggish behaviour, twirling, spiral or erratic movement, lethargy, darkened pigment, eye damage/exophthalmia, haemorrhaging in the mouth, gill damage, white and/or dark nodules on the gills and/or skin, fin rot, haemorrhaging at the base of the fins, distended abdomen, haemorrhaging on the surfaces and muscles, ulcers, and haemorrhaging around the vent (Thompson *et al*., 2004a).

*Vibrio harveyi*, and *Vibrio anguillarum* are the most frequently isolated marine *Vibrio* species (Arias *et al*., 1999; Pujalte *et al*., 1999; Pujalte *et al*., 2003; Frans *et al*., 2011; Chatterjee and Haldar, 2012), having been associated with large-scale losses of larval and juvenile penaeids and also causing several opportunistic diseases to fishes (Hispano *et al*., 1997; Company *et al*., 1999; Diggles *et al*., 2000; Alcaide *et al*., 2001; Liu *et al*., 2003; Zorrilla *et al*., 2003; Chatterjee and Haldar, 2012). Due to the plasticity of *Vibrio* genomes, with frequent horizontal gene transfer events, species boundaries are very narrow in the marine environment (Fraser *et al*., 2007; Chatterjee and Haldar, 2012). Hence, the identification of *Vibrio*-related species isolated from the marine environment is sometimes difficult (Chatterjee and Haldar, 2012). *Vibrio harveyi* is one of the most commonly isolated marine *Vibrio* species, and can easily be found both as free-living or associated to the intestinal microbiota of marine animals (Ramesh *et al*., 1990; Makemson and Hermosa Jr, 1999; Pujalte *et al*., 2003). Moreover, *V. harveyi* is the dominant heterotrophic species in western Mediterranean seawater and marine bivalves during the warm season (Ortigosa *et al*., 1994; Arias *et al*., 1999; Pujalte *et al*., 1999; Pujalte *et al*., 2003). Although not included among the main classical fish pathogens, *V. harveyi* has been related to several opportunistic infections of ornamental or edible cultured fish in the last decade (Kraxberger-Beatty *et al*., 1990; Saeed, 1995; Hispano *et al*., 1997; Company *et al*., 1999; Pujalte *et al*., 2003), and recent reports confirm the virulence of some strains for gilthead sea bream, silver mullet, salmon and seahorse (Balebona *et al*., 1998; Álvarez *et al*., 1998; Zhang and Austin, 2000; Alcaide *et al*., 2001; Pujalte *et al*., 2003).

*Vibrio anguillarum* is a Gram-negative bacterium that causes haemorrhagic septicaemia in fish, a disease that leads to great economic losses in fish farming worldwide (Hong *et al*., 2007). The causative agent, *Vibrio anguillarum*, was initially isolated by Canestrini (1893) (Gonzalez *et al*., 2003) and since the first identification, vibriosis has been described in anadromous and catadromous species (Toranzo and Barja, 1990; Austin and Austin, 1999; Pedersen *et al*., 1999a; Gonzalez *et al*., 2003) and is reported to produce disease in more than 48 fish species (Austin and Austin, 1999; Gonzalez *et al*., 2003). Among the 23 different O-serogroups described for *Vibrio anguillarum*, only serogroups O1, O2 and O3 are important as the causative agent of mortalities in farmed and feral fishes, with the remaining serogroups considered to comprise mainly environmental strains isolated from water and sediment (Sørensen and

Larsen, 1986; Pedersen *et al*., 1999b; Gonzalez *et al*., 2003). Microflora associated with healthy and diseased sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) larvae were also investigated (Grisez, 1997; Grisez *et al*., 1997; Pedersen *et al*., 1999a; Pedersen *et al*., 1999b; Gonzalez *et al*., 2003) and was demonstrated that *V. anguillarum* constituted a significant part of the intestinal microflora of these larvae during feeding with rotifers and that *V. anguillarum* was dominant during outbreaks of disease, causing high mortalities among the larvae (Pedersen *et al*., 1999b).

These bacterial pathogens are important etiological agents that hamper aquaculture production sharing common morphological characteristics and cause similar clinical signs in diseased fish, making the rapid diagnosis of multiple and secondary infections through culture difficult (Zhang *et al*., 2014).

## 4. PCR diagnosis

The rapid development of molecular biological techniques offers significant advantages for workers involved in fish disease diagnosis (Chatterjee and Haldar, 2012), so in recent years, the number of publications describing new molecular techniques or methods has increased significantly (Hirono *et al*., 1996; Brasher *et al*., 1998; Romalde *et al*., 1999; Cerdà-Cuéllar *et al*., 2000, Botella *et al*., 2002; Conejero and Hedreyda, 2003; Avendaño-Herrera *et al*., 2004; Thompson *et al*., 2005; Bramha Chari and Dubey, 2006; Bauer and Rørvik, 2007; Beaz-Hidalgo *et al*., 2008; Amagliani *et al*., 2009; Espiñeira *et al*., 2010; Altinok, 2011; Chang *et al*., 2012; Ransangan and Lal, 2013; Castro *et al*., 2014; Zhang *et al*., 2014). Individual PCR assays have been developed for detection and identification of the fish pathogens (Altinok *et al*., 2008). There has been much interest in the development of specific PCR protocols, many of them based on the amplification of 16S rRNA genes, for detecting a variety of Gram negative and Gram positive bacterial fish pathogens in fish samples and complex substrates (Brown *et al*., 1994; Cunningham, 2002; Romalde and Toranzo, 2002; Beaz-Hidalgo *et al*., 2008). However, a large number of individual PCR reactions would be necessary if single primer sets were used to screen a large number of clinical samples, resulting in a relatively costly and time-consuming process (Altinok, 2011).

Currently, fish photobacteriosis diagnosis is carried out through standard microbiological methods, which are time-consuming and laborious, relying on pathogen culture and isolation steps and the complete protocol always includes biochemical and serological confirmation, leading to an extension of the time needed for the final diagnosis (Amagliani *et al*., 2009). The diagnosis of bacterial infection in aquatic animal has been based on the microbiological analysis using bacteriological culture, morphological characteristic and biochemical tests. Biochemical tests often lead to misinterpretation of results because of strain metabolic variability (Thyssen *et al*., 1998; Botella, *et al*., 2002; Amagliani *et al*., 2009). Additional drawbacks are related to the slow growth of this species and its inhibition by other fast-growing bacteria present in the same samples (Romalde, 2002; Amagliani *et al*., 2009) and to the viable, but non-culturable form that Phdp can also assume (Magariños *et al*., 1994). Several authors have reported that 16S rRNA gene does not provide a sufficient phylogenetic resolution at the species level for *Vibrio* or *Photobacterium* species (Osorio and Klose, 2000; Thompson *et al*., 2005; Martins *et al*., 2015). According to previous studies (Osorio *et al*., 1999; Osorio and Klose, 2000) the subspecies *Photobacterium damselae* subsp. *damselae* and *Photobacterium. damselae* subsp. *piscicida* have 100% homology between sequences of 16S rRNA gene and only 91% homology between sequences of toxR gene (Martins *et al*., 2015).

An array of phenotypic and genomic techniques has become available for the identification of *Vibrio* species in the last three decades (Vandamme *et al*., 1996; Rademaker *et al*., 1998; Savelkoul *et al*., 1999; Olive and Bean, 1999; Rademaker *et al*., 2000; Dijkshoorn *et al*., 2001; Gurtler and Mayall, 2001; Van Belkum *et al*., 2001; Chatterjee and Haldar, 2012).

*V. alginolyticus* has been reported to easily out number other *Vibrio* species in environmental samples (Oliver and Kaper, 2001; Ransangan and Lal, 2013), which may cause the detection of other bacteria difficult (Ransangan and Lal, 2013). Differentiation of these bacteria using phenotypic characterization and 16S rRNA sequencing is also difficult because of high genome homology among *Vibrio* species (Thompson *et al*., 2005; Ransangan and Lal, 2013). Recently, several genes of *Vibrio anguillarum*, such as hemolysin, *angE*, *rpoN*, and 16S rRNA genes, were cloned using PCR (Wiik *et al*., 1995; Hirono *et al*., 1996; Gonzalez *et al*., 2003; Liu *et al*., 2004; Demircan and Candan, 2006). Sigma factor σ54 is responsible for regulating the genes providing coordination between carbon and nitrogen fixation in bacteria (Demircan and Candan, 2006). This factor is also necessary for decarboxylic acid transportation, toluene and xylene catabolism, hydrogenase biosynthesis, and the translation of gene coding for flagella production and nitrogen fixation (Merrick, 1993; Demircan and Candan, 2006). O'Toole *et al*. (1997) were the first group to sequence the 2218 bp rpoN gene; then Gonzalez *et al*. (2003) amplified the 519 bp portion of this gene to identify *Vibrio anguillarum* in fish blood and other tissues (Demircan and Candan, 2006). However, extensive database comparisons demonstrate that differences in the 16S gene sequence between *V. anguillarum* and closely related species are insufficient to warrant the design of species-specific PCR primers using that gene as a target (Gonzalez *et al*., 2003).

Among the currently available *V. anguillarum* gene sequences in the EMBL database, Gonzalez *et al*. (2003) selected *rpoN* gene coding for the cellular sigma factor σ54 as a PCR target. As a housekeeping gene, *rpoN* is expected to be present in all *V. anguillarum* isolates (Gonzalez *et al*., 2003). Moreover, regions of high sequence variability are found in this gene compared to the homologous genes in other *Vibrio* species (Gonzalez *et al*., 2003).

Considering the damages that these bacteria can bring about to fish and human, a rapid, simple, simultaneous and low cost detection method is necessary (Ransangan and Lal, 2013). Compared to traditional methods, these molecular techniques can avoid problems that are inherent in investigating organisms for which no culture medium, cell lines (for viruses) or detection method is available (Lievens *et al*., 2011).

Although this bacterium has been reclassified as *Listonella anguillarum* based on 5S rRNA gene sequence analysis, it is still commonly referred to as *Vibrio anguillarum* (MacDonell and Colwell, 1984; Hong *et al*., 2007). Recently, putative virulence genes of *V. anguillarum* were identified by random genome sequencing (Rodkhum *et al*., 2006a; Hong *et al*., 2007). Di Lorenzo *et al*. (2003) have also determined the complete sequence of the virulence plasmid pJM1 from *V. anguillarum*, which affects marine fish (Hong *et al*., 2007). The phylogenetic relationships between *V. anguillarum* and other *Vibrio* species have been reported, based on comparisons of the DNA sequences of PCR amplicons generated using specific primers that target the recA and 16S rRNA genes (Dorsch *et al*., 1992; Kita-Tsukamoto *et al*., 1993; Urakawa *et al*., 1997; Thompson *et al*., 2004b; Hong *et al*., 2007). However, these genes are not useful in discriminating between closely related strains, due to the very high degrees of sequence identity among these strains (Hong *et al*., 2007). Gonzalez *et al*. (2003) have demonstrated that the annealing temperature is very important in detecting the PCR product using specific primers for the rpoN gene (Hong *et al*., 2007). The expected band also appeared at the normal annealing temperature with *Vibrio ordalii*, which is known to be a very difficult strain to differentiate from *V. anguillarum* (Hong *et al*., 2007). A multiplex PCR has been reported for the specific detection of *V. anguillarum* using primers that target five hemolysin genes (Rodkhum *et al*., 2006b; Hong *et al*., 2007). This method also fails to discriminate *V. ordalii* from *V. anguillarum* reliably, and, consequently, there remains a need for specific primers for PCR detection of *V. anguillarum* strains (Hong *et al*., 2007).

## 5. m-PCR

When multiple bacterial pathogens are likely to occur, as in the aquatic environment, amplification of multiple target genes in a single reaction mixture is possible with the multiplex PCR (m-PCR) method (Brasher *et al*., 1998, Del Cero *et al*., 2002, Panicker *et al*., 2004; Panangala *et al*., 2007), thus reducing cost, time and effort without compromising the test utility (Panangala *et al*., 2007). Although simultaneous detection of several pathogens with a multiplex PCR (mPCR) has been widely applied to the detection of multiple viruses and bacteria in clinical specimens, this approach has not been widely used in the detection of fish pathogens (Osorio *et al*., 2000; Del Cerro *et al*., 2002; Mata *et al*., 2004; Altinok *et al*., 2008; Castro *et al*., 2014), and reports of applications of these techniques on a routine basis in diagnostic laboratories are few (Chatterjee and Haldar, 2012).

### 5.1 *State of the art*

Several attempts to develop methods for the rapid and accurate diagnosis of edwardsiellosis have been made, including PCR-based methods (Castro *et al*., 2014). Of these, a PCR protocol employing the gene *etfD* (which encodes the upstream region of the fimbrial gene) reported by Sakai *et al*. (2007) was shown to be the most rapid and sensitive method for the accurate detection of *E. tarda* in infected fish (Castro *et al*., 2014).

The plasmid content has proved to be very different depending on the geographical origin of subsp. piscicida strains (Magariños *et al*., 1992, Magariños *et al*., 1996), but Osorio *et al*. (2000), proved that, regardless of geographical origin and source of isolation, strains of *Photobacterium damselae* subsp. *piscicida* show one band with a molecular weight of 267 bp corresponding to *ureC* gene.

In 2006, Bramha Chari and Dubey developed PCR-based identification methods for *V. harveyi* targeting a partial 16S rRNA gene (Bramha Chari and Dubey, 2006; Chatterjee and Haldar, 2012). Fukui and Sawabe modified the method by developing a one step colony PCR targeting the same 16S rRNA gene to identify pathogenic *V. harveyi* from aquaculture settings (Fukui and Sawabe, 2007; Chatterjee and Haldar, 2012). Similarly, Conejero and Hedreyda in 2003 targeted the *tox*R gene for identification of *V. harveyi* from aquaculture systems (Conejero and Hedreyda, 2003; Chatterjee and Haldar, 2012). However, the most precise method to identify *V. harveyi* along with *V. campbellii* and *V. parahaemolyticus* was developed by Haldar *et al*. in 2010, using multiplex PCR; this method was so accurate that the individual detection limit of all three target species ranged from 10 to 100 cells per PCR tube, using primer concentrations of 0.25 to 0.5 µmol/l (Haldar *et al*., 2010; Chatterjee and Haldar, 2012).

The main drawback of molecular methodology is that it does not discriminate between DNA from alive and dead microorganisms (Espiñeira *et al*., 2010). Other methodological approaches are focused on the detection of mRNA, because this molecule is less stable than DNA and therefore will not be detected in a sample unless there are viable microorganisms that synthesize it during the enrichment phase (Birmingham *et al*., 2008; Espiñeira *et al*., 2010).

### 5.2 Tool Development

The ability to determine bacterial pathogens using multiplex PCR method was reported dependent to the target genes (Ransangan and Lal, 2013). However, success of this method depends on the selection of target gene, which should be species-specific, widely distributed and also stable in the genome (Chatterjee and Haldar, 2012). Fortunately, both housekeeping and virulent genes can equally serve as good targets for multiplex PCR amplification (Ransangan and Lal, 2013). However, Bauer and Rørvik (2007) also showed that single gene (*ToxR*) can be used as the target PCR amplification of similar bacterial species (Ransangan and Lal, 2013). Nevertheless, virulent genes could serve a better target for multiplex PCR amplification because of their divergence and because they are highly conserved among *Vibrionaceae* (Osorio and Klose, 2000; Ransangan and Lal, 2013).

The 16S rRNA gene (about 1,500 bp in length) consists of highly conserved regions and is present in almost all bacteria which may reveal deep-branching (e.g., classes, phyla) relationships, while variable regions may be demonstrated to be useful in discriminating species within the same genus (Chatterjee and Haldar, 2012). This feature has prompted researchers to use 16S rRNA both as a phylogenetic marker and as an identification tool (Wiik *et al*., 1995; Chatterjee and Haldar, 2012). It has been demonstrated that different selective media are not quite selective or species-specific (Chatterjee and Haldar, 2012). Detection of different marine bacteria on selective media and subsequent colony hybridization with species-specific probes (probe is a fragment of DNA or RNA of variable length, used in DNA or RNA samples to detect the presence of nucleotide sequences), based on variable target regions of the 16S rRNA and other specific genes have been evaluated as an alternative fast screening tool for identification of marine bacteria (Martínez-Picado *et al*., 1996; Cerdà-Cuéllar *et al*., 2000; Cerdà-Cuéllar and Blanch, 2002; Tanaka *et al*., 2002; Sloan *et al*., 2003; Chatterjee and Haldar, 2012).

However, there is nearly 100% 16S rRNA gene homology among many closely related bacterial species, *viz*. *V. scophthalmi* and *V. ichthyoenteri,* thus there is a significant possibility of cross-hybridization and misidentification of closely related species (Cerdà-Cuéllar *et al*., 2000; Chatterjee and Haldar, 2012).

It is not easy to incorporate more than six primer sets because of the cross-reaction in m-PCR, and the challenges inherent in size discrimination among PCR products by conventional electrophoresis (Warsen *et al*., 2004; Chang *et al*., 2012).

## 6. Test organism

The species belonging to the Scianidae family and selected for this present experiment is *Argyrosomus regius* known as Meagre. It is a good candidate for the diversification on commercial aquaculture in Mediterranean and Eastern Atlantic for its good flesh, easy management and high growth rate (Jiménez *et al*., 2005; El-Shebly *et al*., 2007; Roo *et al*., 2010; Velazco-Vargas *et al*., 2013).

Meagre (*Argyrosomus regius)* aquaculture has recently developed, starting in the mid-1990s in Southern France, and is much less advanced than for developed farm fish species such as sea bass, sea bream or turbot (Martínez-Llorens *et al*., 2011). The production of meagre (*A. regius*) began in the second half of the 90s, following an agreement between Italian and French producers, which resulted in the first commercial production in 1997 in France (Amoedo, 2011), spreading, in the years following, to other Mediterranean countries and increasing its production rapidly.

Being a eurihaline species has an easy adaptation to different environments, including growth in earthen ponds with brackish water, also tolerating the imprisonment as demonstrated by its presence in large aquariums and achieving high growth rates and good food conversion levels (Calderón *et al*., 1997; Shepherd *et al.*, 2002; Amoedo, 2011). The organoleptic characteristics of aquaculture meagre are considered very good, characterized by a high protein content and low lipid content (1.5 to 4%) compared to other species of fish, tolerating long periods of cold (1ºC with ice cover) on storage conditions (with a shelf life about 9 days of refrigeration), characteristics that give the fish farming meagre the category of a product of excellence (Poli *et al*., 2003; Amoedo, 2011). This species does not have significant pathological manifestations, being parasites like *Amyloodinium ocellatum, Gyrodactylus* spp. and the bacterium *V. anguillarum* its major pathogens, having already been developed treatment for those, so the control the crop density is the primary method of prevention (Amoedo, 2011). Meagre could be interesting for aquaculture: high flesh quality and flavour, high commercial value over 2 kg, rapid growth between 16 and 20°C, high tolerance to salinity, excellent biological characteristics, because they withstand captivity perfectly, with high growth and good feed conversion ratio (Calderón *et al*., 1997; Martínez-Llorens *et al*., 2011). Juveniles (Age 1) eat small demersal fish and crustaceans (mysids and shrimp) and when they reach 30 to 40 cm, they feed on pelagic fish and cephalopods (Calderón *et al*., 1997; Martínez-Llorens *et al*., 2011). Meagre production has been increasing in recent years with a significant production in 2006, as a result of the achievement of reproduction in captivity (Martínez-Llorens *et al*., 2011). According to Apromar (2008), meagre production in that year was around 845 tm with which a new growth is observed compared with 2005, which is indicative of the establishment and the importance of its production (Martínez-Llorens *et al*., 2011).

The sciaenid meagre (*Argyrosomus regius)* is found in the Mediterranean and Black Sea and along the Atlantic coasts of Europe and the west coast of Africa (Poli *et al*., 2003). Meagre lives in inshore and shelf waters, close to the bottom or near the surface (depth range 15–200 m); it also enters estuaries and coastal lagoons (Chao, 1986; Griffiths and Heemstra, 1995; Poli *et al*., 2003). The fish can reach over 50 kg in the wild, the largest size recorded being 182 cm total length and 103 kg of body weight (Quéro and Vayne, 1987; Poli *et al*., 2003). Its flesh quality is much appreciated (*regius* for royal quality of flesh) (Poli *et al*., 2003). In 2003 only a few attempts have been made at farming this species in Europe, between these, only one French farm (Les Poissons du Soleil), with hatchery, nursery and on growing facilities, has succeeded in the artificial reproduction and rearing of meagre and just two French and two Italian marine farms have intensively grown fry up to market sizes (Poli *et al*., 2003).

## 7. Aims of the study

The aim of the present study was to develop a multiplex-PCR tool for detection of *Edwardsiella tarda*, *Photobacterium damselae* subsp. *piscicida*, *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio anguillarum* in tissue and blood samples of diseased fish.

# Chapter II.

## Development of an m-PCR tool for detection of four bacterial pathogens on Aquaculture

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## 1. Introduction

Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovation (Toranzo *et al*., 2005). Bacterial diseases are one major problem causing fish mortality and economic loss in aquaculture (Ransangan and Lal, 2013). Fast detection of these pathogens is important for management and control of disease outbreaks (Martins *et al*., 2015).

Considering the damages that some bacteria can cause to humans and fish, it is necessary a fast, simple, simultaneous (detection of different pathogens in one single reaction at the same time) and low cost detection method being its development the primary objective of this study.

We have selected as development targets of a multiplex PCR tool the following pathogens: *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi* and still *Edwardsiella tarda* and *Photobacterium damselae* subsp. *piscicida*. *Vibrio* species under optimum temperature and salinity conditions arise in high amounts in aquatic organisms (Espiñeira *et al*., 2010) and *E. tarda* has recently become an emerging bacterial pathogen in aquaculture (Castro *et al*., 2014), affecting a wide range of cultivated species. It is associated with life-threatening sepsis and infections in various animals, including humans (Castro *et al*., 2014). Members of *Photobacterium damselae* species have been described also as emerging pathogens of fish in aquaculture systems (Martin *et al*., 2015), causing sepsis (Osorio *et al*., 2000). Pathogenicity, frequency of relapses and their severity, determined the choice of these species. The development of this molecular tool is also important because it covers pathogens that affect a wide range of cultivated organisms such as bivalve mollusks, crustaceans and fish, decreasing costs for companies that already have different types of cultures or it may be a mean to encourage aquaculture companies to diversify their production.

## 2. Materials and Methods

### 2.1. Bacterial strains and culture conditions

In total, five bacterial species were used in this study: *Edwardsiella tarda* (ACC 36.1), *Photobacterium damselae* subsp. *piscicida* (AQP 17.1), *Vibrio harveyi* (DSM 19023), *Vibrio anguillarum* (AQV 55.1) and *Vibrio alginolyticus* (CECT 521). All bacteria were cultured on Tryptone Soya Broth (TSB; Himedia), supplemented with 1.5% of sodium chloride (NaCl; Panreac), except for *E. tarda* that was with only 1%, and growth curves were established.

### 2.2. DNA extraction from bacterial pure culture and confirmation

Bacterial DNA extraction was performed with NZY Tissue gDNA isolation kit (nzytech, genes & enzymes) for 1 ml of pure culture.

For confirmation, the PCR reactions (20 µL) were set with 10 µL of Master Mix (NZYTaq 2x Colourless Master Mix), 1 µL $MgCl_2$, 1 µL of each primer, 5.5 µL of sterile distilled water and 1.5 µL of template DNA. The PCR reaction was initiated with a denaturation of 5 minutes at 95ºC, followed by 45 cycles of denaturation for 30 seconds at 95°C, annealing 30 seconds at 51°C and extension for 1 minute at 72°C. The final extension step consisted of 10 minutes at 72°C. After addition of the dye RED safe amplified products were separated on agarose gel at 1.5% (w/v) with 1x TAE buffer and visualized in UV transilluminator. The horizontal electrophoresis ran for 60 minutes at a voltage of 70 V.

Bacterial DNA extracted was quantified with a Nanodrop 2000 Spectrophotometer (Thermo scientific) in ng/µl.

### 2.3. PCR primers used in this study

Primers used in this study are listed in Table I with respective target bacteria, name, forward and reverse sequences, melting temperatures (ºC), expected size band (bp) and reference of the author. EtdF primer was design with the help tool Primer-Blast from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye *et al*., 2012).

**Table I - Characteristics of the primers used in this study and their references.**

| Target bacteria | Primer name | Sequences (5'-3') | Melting temperature (ºC) | Expected size (bp) | References |
|---|---|---|---|---|---|
| *E. tarda* | EtdF | AGCGCAGCTAACGGTAAAGT | 57 | 426 | This study |
| | EtfA_R | TGTAACCGTGTTGGCGTAAG | 55 | | Sakai et al. (2007) |
| *Phdp* | Car1 | GCTTGAAGAGATTCGAGT | 49 | 267 | Osorio *et al* (2000) |
| | Car2 | CACCTCGCGGTCTTGCTG | 59 | | Osorio *et al* (2000) |
| *Phdp* | Ure5 | TCCGGAATAGGTAAAGCGGG | 57 | 448 | Osorio *et al* (2000) |
| | Ure3 | CTTGAATATCCATCTCATCTGC | 51 | | Osorio *et al* (2000) |
| *V. alginolyticus* | ValF | CTCTCCCAATTCAGCCCTCTA | 56 | 773 | Ransangan and Lal (2013) |
| | ValR | GACTCTTCACAACAGAACTC | 51 | | Ransangan and Lal (2013) |
| *V. anguillarum* | rpoN-ang5 | GTTCATAGCATCAATGAGGAG | 51 | 519 | Demircan and Candan (2006) |
| | rpoN-ang3 | GAGCAGACAATATGTTGGATG | 51 | | Demircan and Candan (2006) |
| *V. harveyi* | VhF | ACGCTTGATGGCTACTGGTGGAG | 61 | 606 | Ransangan and Lal (2013) |
| | VhR | CTTCGCACCTGCATCGG | 57 | | |

### 2.4. m-PCR analysis

Multiplex-PCR reactions were tested using NZYTaq polymerase, variable annealing temperatures (49ºC, 51ºC, 53ºC and 55ºC) and 0.125 µM of each primer concentrations. Amplification conditions were: 5 min. at 95ºC, 45 cycles (95ºC, 30s; 50±10ºC, 30s; 72ºC, 1 min.) and finally at 72ºC for 7 min.

Was also performed an m-PCR reaction with variable concentrations of $MgCl_2$ (2, 4, 6 and 8 mM) maintaining annealing temperatures and conditions mentioned above.

All reaction products were analyzed by electrophoresis in agarose gel (1,5%), using a 250 bp ladder (Alfa Aesar) as a molecular weight marker.

### 2.5. Specificity and sensitivity of m-PCR assay

The specificity of the primers was tested using purified bacterial DNA of the respective strains. For a multiplex-PCR conditions (25 µL) we added 12.5 µL of Master Mix (NZYTaq 2x Colourless Master Mix), 1.25 µL $MgCl_2$, 1 µL of each primer, 4.45 µL of sterile distilled water and 2 µL of mixture of different template DNA (with double quantity of *E. tarda* purified DNA). Amplification conditions were: 5 min. at 95ºC, 45 cycles (95ºC, 30s; 51ºC, 30s; 72ºC, 1 min.) and finally at 72ºC for 7 min.

Sensitivity was tested with eight successive dilutions of a mixture of the four bacterial pathogens purified DNA from a known concentration of 64.6 µg/µL for *E. tarda*; 89.5 µg/µL for *V. alginolyticus*; 110.8 µg/µL for *V. anguillarum* and 24 µg/µL for *V. harveyi*. Multiplex – PCR reaction (20 µL) added 10 µL of Master Mix (NZYTaq 2x Colourless Master Mix), 1 µL $MgCl_2$, 0.25 µL of each primer (except for *E. tarda* that was 0.5 µL of each forward and reverse primers), 3.5 µL of sterile distilled water and 3 µL of mixture of different template DNA. Amplifications conditions were the same used for specificity testing.

### 2.6. Experimental fish infection

After bacteria growth curves were established, the applicability of the mPCR protocol in infected fish was determined through the inoculation of batches of three meagre (average 30-36 g) with 0.1ml of a suspension of each bacteria at a concentration of $10^6$ $CFUml^{-1}$. Three batches of fish were inoculated for each bacterium separately. As the negative control, three batches of fish were inoculated with sterile Hank's Balanced Salt Solution (HBSS) and maintained under the same conditions as the experimentally infected fish. The fish were maintained at equal density of 6 $kgm^{-3}$ with continuous aeration and a water temperature of 22±1ºC. One week post-infection, the kidney, liver, spleen and blood samples were collected from all fish and kept at -80ºC. Classical microbiology analysis was also performed to the liver, kidney and spleen samples.

### 2.7. Tool validation and *spiking* on tissues

When the tool was validated and the conditions set, the total DNA from tissues and blood collected were extracted with DNeasy Blood & Tissue Kit (QIAGEN) and m-PCR was performed in these conditions (20 µL) added 10 µL of Master Mix (NZYTaq 2x Colourless Master Mix), 2 µL $MgCl_2$, 0.5 µL of each primer, 1 µL of sterile distilled water and 3 µL of tissue total DNA. Amplification conditions were: 5 min. at 95ºC, 45 cycles (95ºC, 30s; 51ºC, 30s; 72ºC, 1 min.) and finally at 72ºC for 7 min.

To make it possible to predict the behavior of this tool in opportunistic infection situation in which they occur one or more pathogens, pathogens were cultured, as already mentioned above. After homogenization with 100 µl of Phosphate Buffered Saline (PBS) by *repeated pipetting* of each sample (0.1 to 0.2g) of kidney, liver and spleen of a healthy meagre - previously analyzed with classical microbiology - the samples were spiked with 100 µl of the mixture of the five different pathogens with a concentration of 340 $CFUml^{-1}$ for *E. tarda* (exponential phase), 410 $CFUml^{-1}$ for *V. alginolyticus* (exponential phase, almost in stationary phase), 810 $CFUml^{-1}$ for *V. anguillarum* (exponential phase), 740 $CFUml^{-1}$ for *V. harveyi* (exponential phase) and let them incubate at 25ºC during 1h. Finished incubation, total DNA was extracted with DNeasy Blood & Tissue Kit (QIAGEN) and m-PCR was performed along electrophoresis.

## 3. Results

### 3.1. m-PCR analysis

Different annealing temperatures (49ºC, 51ºC, 53ºC and 55ºC) were tested and were obtained four expected size bands (*E. tarda* – 426 bp; *V. anguillarum* – 519 bp; *V. harveyi* – 606 bp; *V. alginolyticus* – 773 bp) at 49ºC, 51ºC and 53ºC and only three bands at 55ºC (*E. tarda* – 426 bp; *V. anguillarum* – 519 bp; *V. harveyi* – 606 bp). For Phdp was no amplification, so the expected band of 267 bp did not show up. We concluded that the temperature of 51°C is better to distinguish the bands of four different pathogens: *E. tarda*, *V. anguillarum*, *V. harveyi* and *V. alginolyticus* (Figure 2.1).

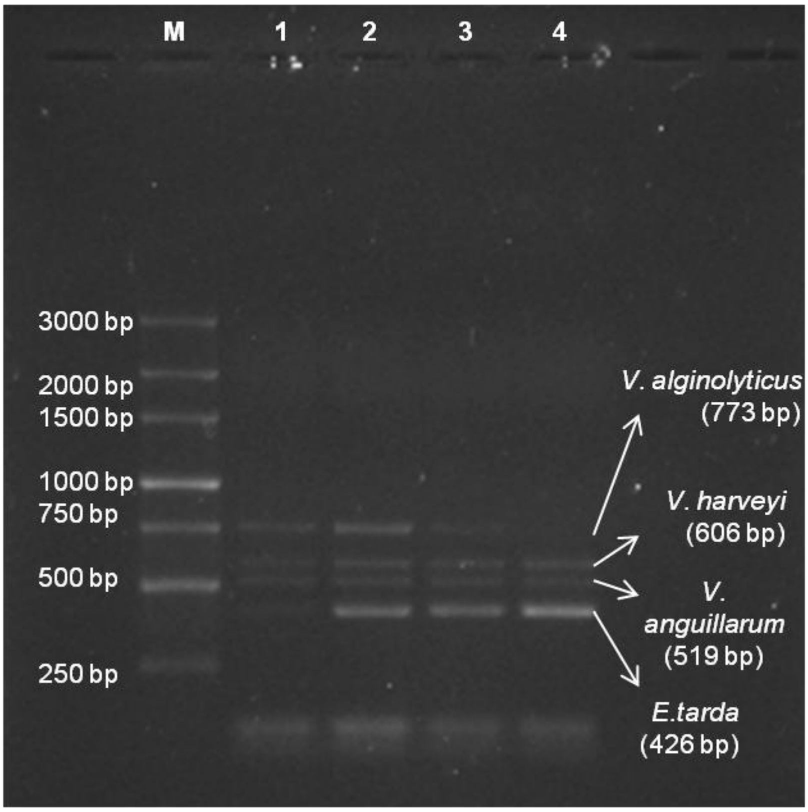

**Figure 2.1 – m-PCR at different annealing temperatures. M – 250 bp ladder; Lane 1 – 49ºC; Lane 2 – 51ºC; Lane 3 – 53°C; Lane 4 – 55ºC.**

Different concentrations of $MgCl_2$ (2 mM, 4 mM, 6 mM and 8 mM) were also tested along the different annealing temperatures mentioned above and the best results obtained were four expected size bands (*E. tarda* – 426 bp; *V. anguillarum* – 519 bp; *V. harveyi* – 606 bp; *V. alginolyticus* – 773 bp). It follows that the temperature of 51°C along with between 4 and 6 mM of $MgCl_2$ is better to distinguish the bands of the four different pathogens: *E. tarda*, *V. anguillarum*, *V. harveyi* and *V. alginolyticus* (Figure 2.2).

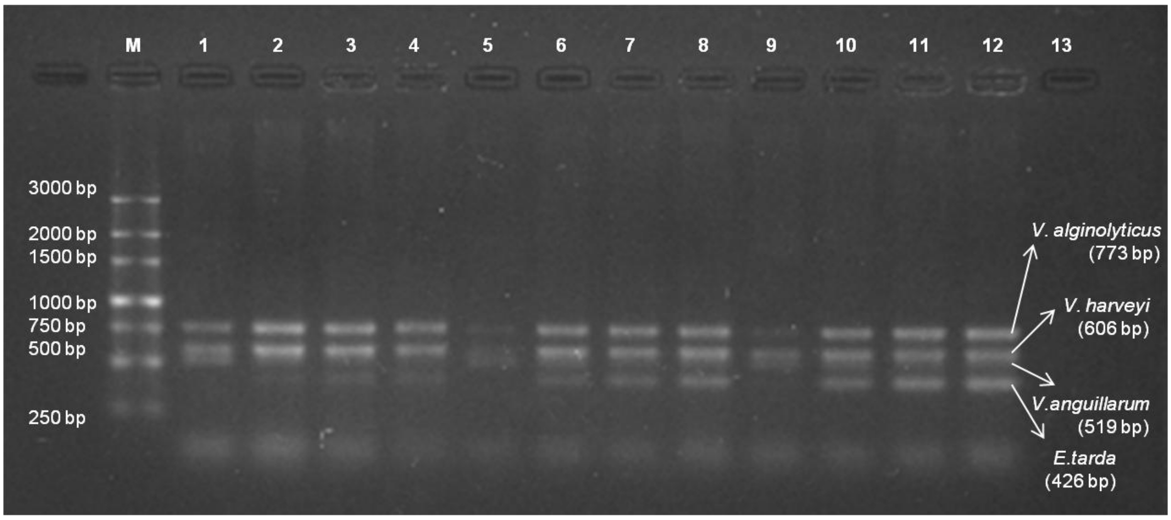

**Figure 2.2 – m-PCR at different annealing temperatures and different concentrations of $MgCl_2$. Lane 1 – 250 bp ladder; Lane 2 - 49ºC, 2 mM $MgCl_2$; Lane 3 – 49ºC, 4 mM $MgCl_2$; Lane 4 – 49ºC, 6 mM $MgCl_2$; Lane 5 – 49ºC, 8 mM $MgCl_2$; Lane 6 - 51ºC, 2 mM $MgCl_2$; Lane 7 - 51ºC, 4 mM $MgCl_2$; Lane 8 - 51ºC, 6 mM $MgCl_2$; Lane 9 - 51ºC, 8 mM $MgCl_2$; Lane 10 - 53ºC, 2 mM $MgCl_2$; Lane 11 - 53ºC, 4 mM $MgCl_2$; Lane 12 - 53ºC, 6 mM $MgCl_2$; Lane 13 - 53ºC, 8 mM $MgCl_2$.**

### 3.2. Specificity and sensitivity of m-PCR assay

The specificity of the primers was tested using purified bacterial DNA of the respective strains, yielding bands at expected sizes (*E. tarda* – 426 bp; *V. anguillarum* – 519 bp; *V. harveyi* – 606 bp; *V. alginolyticus* – 773 bp), which can be identified on agarose gels with clearness and without overlapping sizes, except for Pdhp, that doesn´t show the expected band of 267 bp but a non-specific band over 1500 bp (Figure 2.3).

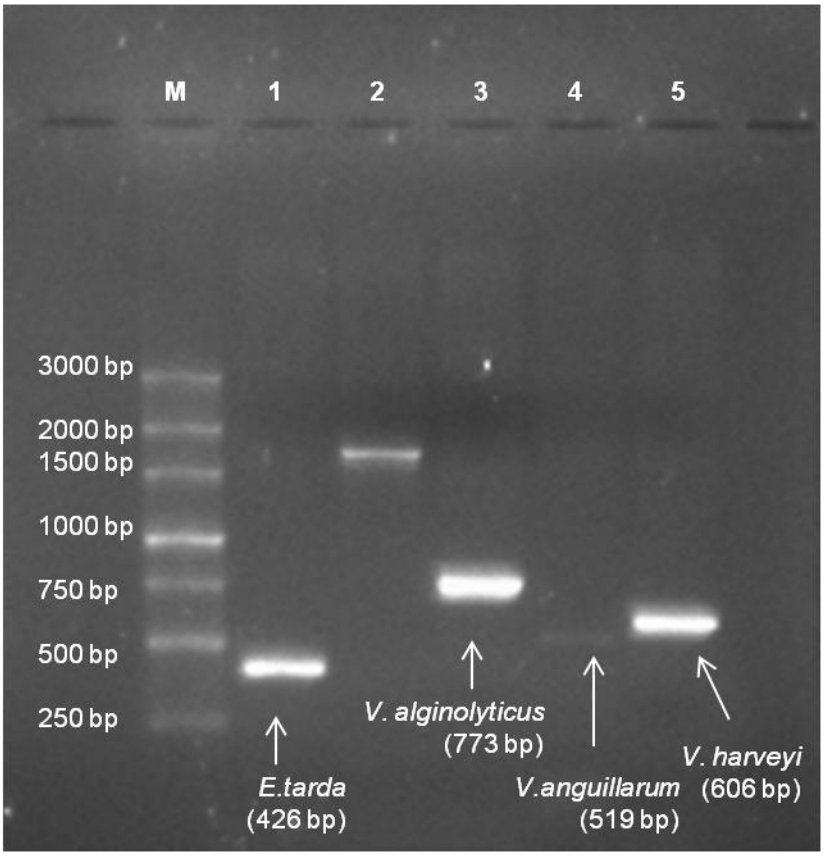

**Figure 2.3 - Amplification of assay primers in monoplex reaction. M - 250 bp ladder; Lane 1 - *E. tarda* (369 bp); Lane 2 - Phdp (expected size 267 bp); Lane 3 - *V. alginolyticus* (773 bp); Lane 4 – *V. anguillarum* (519 bp); Lane 5 – *V. harveyi* (606 bp).**

The detection limit of the multiplex PCR amplification was as low as 0.4 ng/µl for *V. anguillarum*; 0.5 ng/µl for *E. tarda*; 1.5 ng/µl for *V. harveyi* and 5.6 ng/µl for *V. alginolyticus* (Figure 2.4).

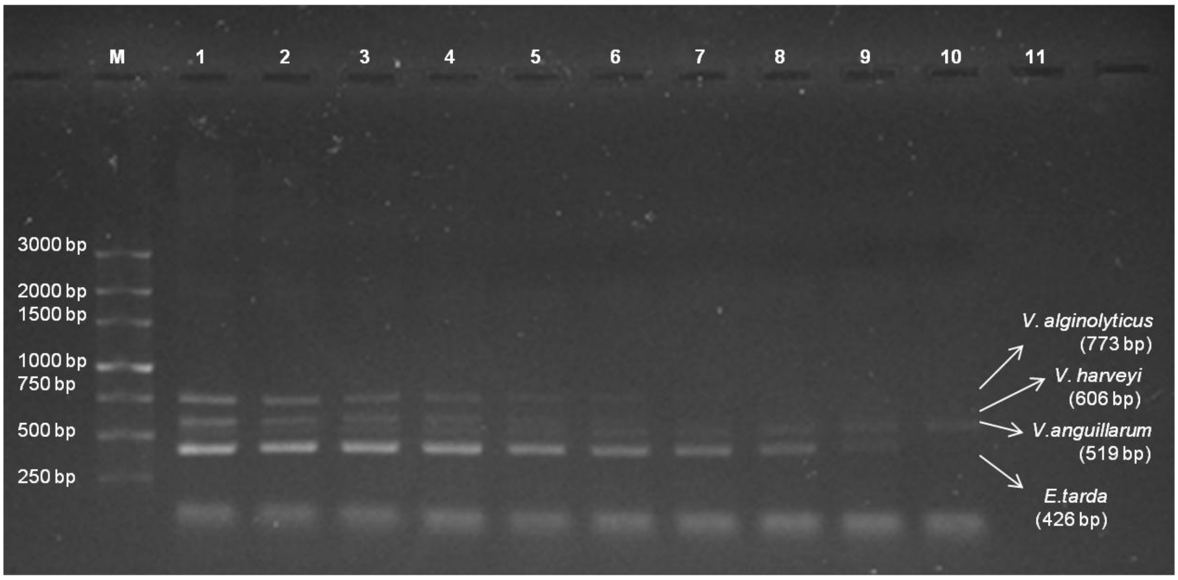

**Figure 2.4 – m-PCR sensitivity test. Lane 1 – 250 bp ladder; Lane 2 – mixture of different DNA (64.6 ng/µl for *E. tarda*; 89.5 ng/µl for *V. alginolyticus*; 110.8 ng/µl for *V. anguillarum*; 24 ng/µl for *V. harveyi*); Lane 3 – mixture of different DNA (32.3 ng/µl for *E. tarda*; 44.8 ng/µl for *V. alginolyticus*; 55.4 ng/µl for *V. anguillarum*; 12 ng/µl for *V. harveyi*); Lane 4 – mixture of different DNA (16.2 ng/µl for *E. tarda*; 22.4 ng/µl for *V. alginolyticus*; 27.7 ng/µl for *V. anguillarum*; 6 ng/µl for *V. harveyi*); Lane 5 - mixture of different DNA (8.1 ng/µl for *E. tarda*; 11.2 ng/µl for *V. alginolyticus*; 13.9 ng/µl for *V. anguillarum*; 3 ng/µl for *V. harveyi*); Lane 6 - mixture of different DNA (4 ng/µl for *E. tarda*; 5.6 ng/µl for *V. alginolyticus*; 6.9 ng/µl for *V. anguillarum*; 1.5 ng/µl for *V. harveyi*); Lane 7 - mixture of different DNA (2 ng/µl for *E. tarda*; 2.8 ng/µl for *V. alginolyticus*; 3.5 ng/µl for *V. anguillarum*; 0.8 ng/µl for *V. harveyi*); Lane 8 - mixture of different DNA (1 ng/µl for *E. tarda*; 1.4 ng/µl for *V. alginolyticus*; 1.7 ng/µl for *V. anguillarum*; 0.4 ng/µl for *V. harveyi*); Lane 9 - mixture of different DNA (0.5 ng/µl for *E. tarda*; 0.7 ng/µl for *V. alginolyticus*; 0.9 ng/µl for *V. anguillarum*; 0.2 ng/µl for *V. harveyi*); Lane 10 - mixture of different DNA (0.3 ng/µl for *E. tarda*; 0.3 ng/µl for *V. alginolyticus*; 0.4 ng/µl for *V. anguillarum*; 0.1 ng/µl for *V. harveyi*); Lane 11 – control of the reaction, without DNA.**

### 3.3. Experimental fish infection

Classical microbiological methods confirmed the presence of *E. tarda* and *V. anguillarum* in kidney, liver and spleen of specimens of infected meagre (*A. regius*).

### 3.4. Tool validation

The m-PCR was applied to kidney, liver and spleen from spiked tissues. As expected, negative controls produced no amplifications (Figure 2.5).

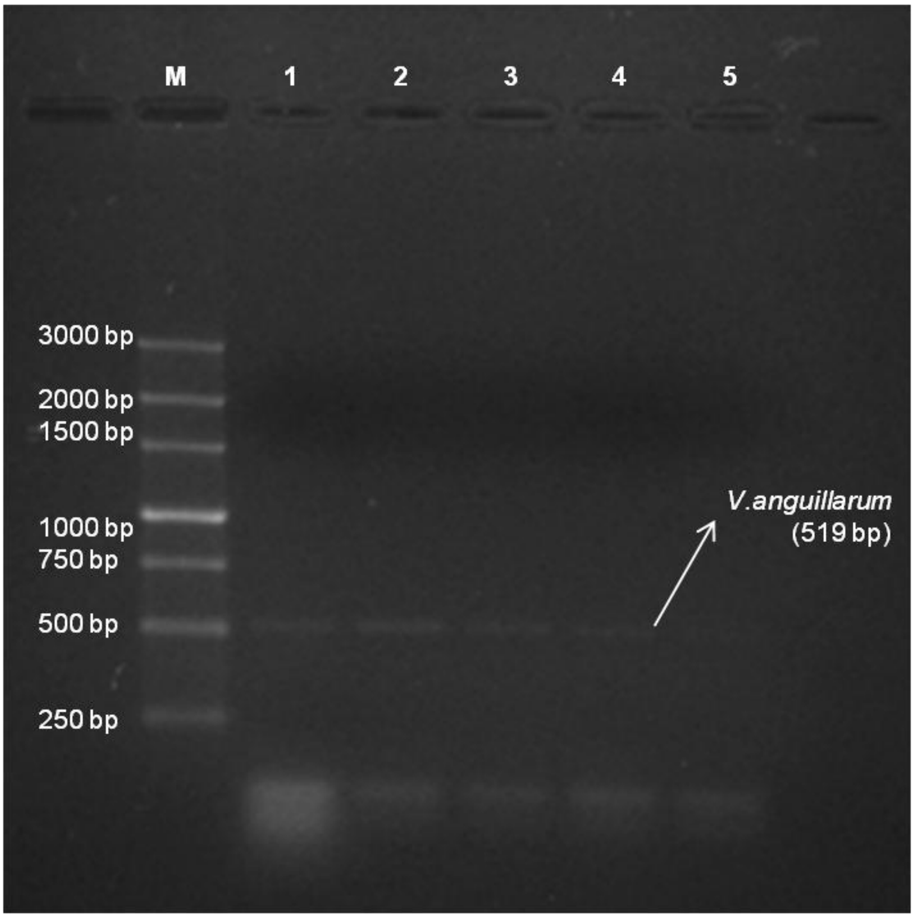

**Figure 2.5 – m-PCR applied to *spiked* spleen, liver and kidney from an apparently healthy meagre (*A. regius*). M – 250 bp ladder; Lane 1 – Spleen tissue spiked with mixture of different test pathogens; Lane 2 – Liver tissue spiked with mixture of different test pathogens; Lane 3 – Kidney tissue spiked with mixture of different test pathogens; Lane 4 – Blood sample from infected symptomatic fish infected with *E. tarda*; Lane 5 – control of the reaction, without DNA.**

This detection method was also applied to liver and blood samples from experimentally infected fish. Negative controls produced no amplifications. All the samples and control reaction presented one band above 500 bp, believed to be a non-specific band resultant from the tissue interference with the multiplex reaction once it appears on the tissue control fish, previously analyzed and confirmed as a healthy fish (Figure 2.6).

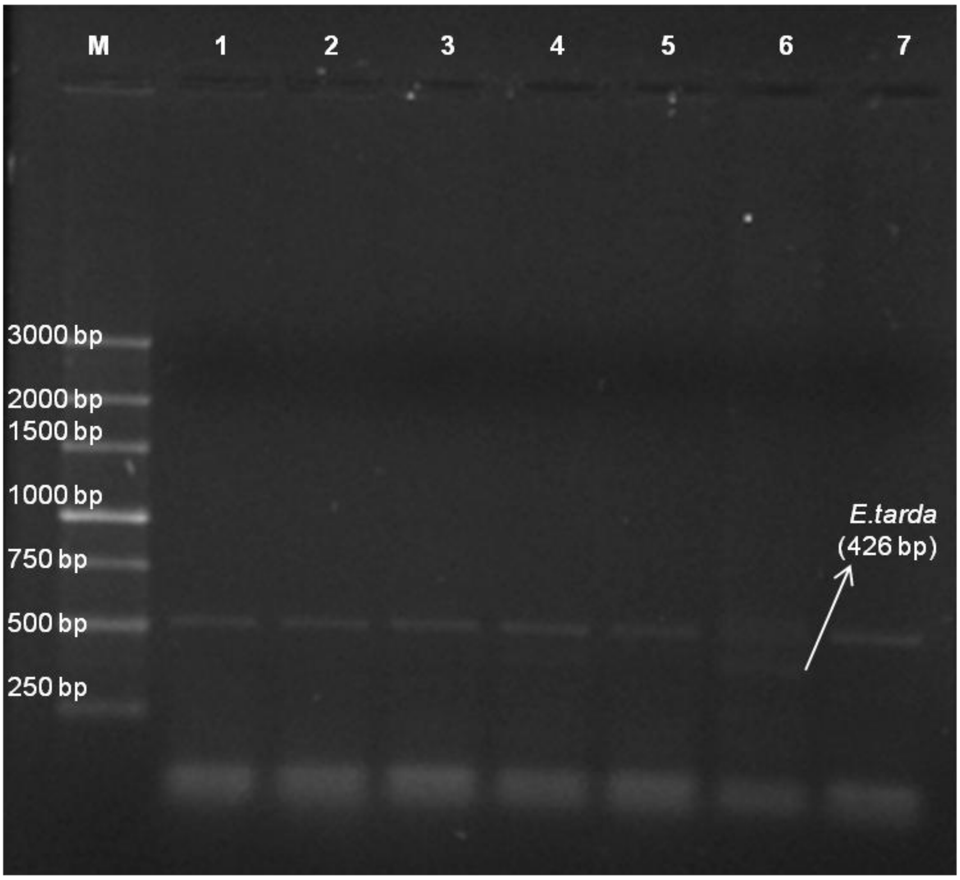

**Figure 2.6 – m-PCR applied to tissue and blood samples from *A. regius*. M – 250 bp ladder; Lane 1 – Tissue from control fish; Lane 2 – Tissue from an infected fish with *E. tarda*; Lane 3 – Tissue from an infected fish with *V. alginolyticus*; Lane 4 – Tissue from an infected fish with *V. anguillarum*; Lane 5 - Tissue from an infected fish with *V. harveyi*; Lane 6 - Blood sample from infected symptomatic fish infected with *E. tarda*; Lane 7 – Control of the reaction, without DNA.**

## 4. Discussion and conclusion

Currently, aquaculture is one of the fastest growing food production systems in the world (Castro *et al*., 2014).

However, the life-history characteristics of the meagre (e.g., longevity, large size and age at maturity, large variability in annual recruitment, formation of spawning aggregations in coastal waters and estuaries) pose significant management and conservation problems for it making the meagre rank high among the world's most vulnerable species (Cheung *et al.*, 2007; Prista, 2013). Despite this, the biology, ecology and fisheries of the meagre are poorly documented, particularly in European waters, and only recently has interest in aquaculture production, management of artisanal fisheries, and the conservation of data-poor fish resources resulted in some direct scientific research on this species (Quéméner, 2002; Prista, 2013).

The variability in fish supply and increase in consumer demand for fish products, alongside the fast growth rate and the good properties of the meat in meagre and putatively good biological properties for growth in captivity (Quéméner, 2002; Prista, 2013) led to a spark in aquaculture production in France during the late nineties (Quéméner, 2002; Monfort, 2010; Prista, 2013). According to Prista (2003), aquaculture production of meagre has expanded to seven other southern European countries, including Portugal, reaching 14 000 t/year and being worth nearly 48 million USD/year (FAO, 2012b; Prista, 2013).

Disease management and assessment of cultured aquatic animals is a major concern in commercial aquaculture (Shi *et al*., 2012). Molecular methods like PCR and m-PCR are always more reliable to overcome such problems, being the last one more efficient due to multiple detection of pathogens in an only reaction.

mPCR is generally thought to be less sensitive than single PCR because of competition for reaction reagents, especially if the assays differ in their amplification efficiencies or one or more of the target organisms is present in high numbers (Tapia-Cammas *et al*., 2011; Castro *et al*., 2014).

Products of various lengths present a challenge for developing optimal PCR conditions (primer annealing temperatures and similar $MgCl_2$ concentrations) (Gonzalez *et al*., 2004). In the present work, several reaction conditions were tested for the development of this tool, including varying annealing temperatures (between 49ºC and 55°C) and the variation in the $MgCl_2$ concentration (from 2 mM to 8 mM). Best results were obtained at 51°C with an $MgCl_2$ concentration between 4 mM and 6 mM where it appears the bands of the four of the five pathogens tested (*E. tarda*, *V. alginolyticus*, *V. anguillarum* and *V. harveyi*) (Figure 2.2).

One of the most critical steps in the study of bacterial fish diseases is the correct identification of the infectious agent (Avendaño-Herrera *et al*., 2004). The primers described here proved to be specific under the conditions assayed both in relation to 4 of the 5 target species (*E. tarda*, *V. alginolyticus*, *V. anguillarum* and *V. harveyi*), with only the specific target species showing amplification in the multiplex reaction (Figure 2.3). For this reason, sensitivity was tested only against these 4 target species and not with Phdp that showed a non-specific band above 1500 bp instead of a specific band of 267 bp.

The detection limit of this tool was determined using purified bacterial DNA and was obtained a result of 0.4 ng/µl for *V. anguillarum*; 0.5 ng/µl for *E. tarda*; 1.5 ng/µl for *V. harveyi* and 5.6 ng/µl for *V. alginolyticus* (Figure 2.4). present in a mixture of these bacterial DNA.

Under intensive aquaculture conditions, healthy-looking fish without clinical signs may carry pathogens, posing a serious risk for the spread of diseases among fish populations (Castro *et al*., 2014). Despite of this, some of the infected experimentally fish displayed the typical signs of vibriosis - gill necrosis, lethargy, darkness of the skin and loss of appetite, although microbiology only resulted for symptomatic sacrificed fish or for the fish that died along the experience which can be explained by the medium weight of the meagre (about 32.68 g), which was could be too big for a $10^6$UFCml$^{-1}$ doses, making it resistant to the pathogens. This can cause the immune system to fight disease and decrease the number of invading cells to a level below the detection tool. From the data presented here, we conclude that the multiplex PCR assay is more sensitive than bacteriological culturing.

Therefore, detection of pathogens in carrier fish is essential for effective disease control (Altinok, 2011). The detection of the pathogen can help in identifying asymptomatic carriers, especially when selecting fish for broodstock development (Kulkarni *et al*., 2011).

The role of bacteria varies from their effect as primary pathogen to that of secondary invader in the presence of other disease agents; they may also serve as a stress factor and predispose fish to other diseases (Badran and Eissa, 1991; Khalil and Abd El-Latif, 2013). Higher concentrations of DNA from 1 pathogen had a noticeable effect on amplification of other bacteria in the multiplex reaction when these were present at very low levels (Altinok, 2011), as can be seen in the validation of the tool with spiked tissues (Figure 2.5) in which *V. anguillarum* overlaps the other three pathogens appearing just one band of 519 bp, specific for this pathogen.

According to Lievens *et al*., until 2011, has not been reported the utility of the system for sensitive detection from complex samples, such as infected tissue. This is probably due to the difficulty inherent to the interference of the tissue in m-PCR reaction creating non-specific bands such the band above 500 bp that appeared in our validation of the tool with infected experimentally tissues (Figure 2.6). Another explanation relates to the fact that similar problems in detecting other fish pathogens in kidney have been attributed to PCR inhibitors (Chase and Pascho, 1998; Avendaño-Herrera *et al*., 2004) and negative results were obtained when using DNA extracted from kidney samples in a single PCR (Avendaño-Herrera *et al*., 2004). Although the detection level with fish tissues and blood samples was lower than that with pure cultures (probably due to the presence of host DNA and undefined inhibitors of the PCR reaction (Wilson, 1997; Gonzalez *et al*., 2003).

Blood testing is preferable to tissue analyses because it does not require the sacrifice of the sampled fish and is suitable for monitoring fish on farms (Gonzalez *et al*., 2003), which corroborate the results obtained in this work in which it is possible to detect the pathogen in blood samples (*E. tarda*) before the fish can show haemorrhages and others signs of disease plus abnormal behaviour.

The results indicate that the multiplex PCR tool might be more suitable for detection of four of the five major pathogens tested (*E. tarda*, *V. harveyi*, *V. anguillarum* and *V. alginolyticus*) in blood samples.

This method allows a diagnosis of edwardsiellosis and vibriosis in one working day after simple blood sampling; thus, it appears more convenient than the classical culture-based methods, which are time consuming and not always certain because of variability in phenotypic characters depending on growth conditions.

# Chapter III.

# Concluding Remarks and Future Perspectives

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In summary, the optimum m-PCR protocol for the detection of *E. tarda*, *V. alginolyticus*, *V. anguillarum* and *V. harveyi* in fish consists of an annealing temperature of 51°C and amplification for 45 cycles using the primers mentioned in table I. The PCR protocol described here has practical application for the rapid and early diagnosis of edwardsiellosis and different types of vibriosis in European meagre (*Argyrosomus regius*). The possibility of performing the assay on blood avoids sacrificing the fish during routine assay procedures. This PCR protocol could potentially be used to monitor these diseases in other species of fish.

Although this molecular methods such PCR and m-PCR can bring some advantages, they have some disadvantages too.

PCR assays may vary in amplification efficiency, due to primer length, nucleotide content, and secondary structure which can cause, in one PCR assay within the multiplex against a target in high concentration might outcompete one or more of the other assays to such an extent that the detection limits for the targets at lower concentrations are affected (Altinok, 2011).

The low detection limit of an mPCR method is a potential problem unless adequate safeguards are used in avoiding sample contamination (Altinok *et al*., 2008).

Oligonucleotide microarray, combining PCR technology with hybridization of the resulting amplification products, and post hybridization image processing have produced extremely powerful tools for pathogen detection, differentiation, and identification (Chang *et al*., 2012), while single PCR does not allow differentiation between alive and dead cells of the pathogens.

Subsequent sequencing, which is a relatively costly and laborious process, is often needed to confirm product identity (Chang *et al*., 2012).

It is not easy to incorporate more than six primer sets because of the cross-reaction in m-PCR, and the challenges inherent in size discrimination among PCR products by conventional electrophoresis (Warsen *et al*., 2004). The new developing method, three oligo (primers + probe) PCR (such as TaqMan® real-time PCR) may overcome the problems (Chang *et al*., 2012). Although this method requires more expensive equipment, is suggested to be used in quantitative gene expression and allele discrimination research. To efficiently screen a complex mixture of sequences from different pathogens, DNA microarray is an excellent candidate (Chang *et al*., 2012). These authors demonstrated a naked-eye reading microarray system targeting 16S rDNA to identify eight common fish pathogens, obviating the need for expensive fluorescence detection facilities.

Recent developments in DNA microarray allow parallel hybridizations to occur on the same surface and permit multiple independent detections (Call *et al*., 2003; Chang *et al*., 2012). In most microarray formats, slides are stained with streptavidin-conjugated fluorophore, and the interaction of the target with specific probes is measured by epifluorescence confocal microscopy using an argon ion laser.

In future studies this tool must be improved through quantification of the sensitivity of the tool for blood samples, this time, by testing it in other important farm species in an assay that should be performed with juvenile, more susceptible to disease.

# Chapter IV.

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