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RESEARCH ARTICLE

Development of a new multiplex-PCR tool for the simultaneous detection of the fish pathogens *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi* and *Edwardsiella tarda*

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Abstract – Disease assessment and management in cultured aquatic animals is a major concern in commercial aquaculture. Disease outbreaks have direct effects on fish production, causing serious economic losses in this industry. This can be overcome by early detection through molecular high sensitivity tools such as PCR (polymerase chain reaction). One of the most critical steps in the study of bacterial fish diseases is the precise identification of the infectious agent. Considering the damage that some bacteria can cause to fish and humans, the development of a rapid detection method for the four target species that demonstrates to be simple, accurate and low cost is an essential step for the prevention and early treatment of these diseases. *Edwardsiella tarda* (ACC 36.1), *Vibrio harveyi* (DSM 19023), *Vibrio anguillarum* (AQV 55.1) and *Vibrio alginolyticus* (CECT 521) were selected as targets of a multiplex PCR tool. The multiplex PCR reactions were performed in various reaction conditions, including different annealing temperatures (between 49 °C and 55 °C) and changes in the $MgCl_2$ concentration from 2 mM to 8 mM. Best results were obtained at 51 °C and $MgCl_2$ concentration from 4 mM to 6 mM. Primers were tested using purified DNA from the respective bacterial strains, yielding bands at expected sizes, which can be identified on agarose gels with clearness and without overlapping sizes. Results indicate that this multiplex PCR tool is suitable for the detection of target pathogens and may, in the future, have extended practical application in commercial aquaculture.

Keywords: Disease diagnostic / Multiplex-PCR / *Vibrio alginolyticus* / *Vibrio anguillarum* / *Vibrio harveyi* / *Edwardsiella tarda*

1 Introduction

Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovation. In nature, fish are continually bathed in an aqueous suspension of microorganisms and most of the members of the normal microflora of water are harmless, however some of these can reveal as bacterial fish pathogens (Chang et al., 2012). 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). The possibility of outbreaks is increased if fish are stressed, as what happens when the water temperature is inappropriate, or in conditions of low dissolved oxygen, high nitrite levels, or high culture densities (Perera et al., 1997; Shoemaker et al., 2000). Healthy looking fish without any clinical signs or lesions can carry pathogens and create a serious risk for spread of contagious diseases in fish

populations (Onuk et al., 2010). Traditionally, disease diagnosis is obtained by culturing bacteria on agar plates followed by phenotypic and serological characterization of the pathogen, or by histological examination. Biochemical tests, DNA homology, and protease variability techniques have also been extensively used, but these techniques have major disadvantages, such as the need for initial isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen. Molecular techniques have slowly established a place in the diagnosis of disease in aquaculture (Chatterjee and Haldar, 2012), by overcoming the problems related to the sensitivity and specificity of pathogen detection and identification (Altinok, 2011).

We have selected as development targets of a multiplex PCR tool the following pathogens: *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi* and *Edwardsiella tarda*. *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 (Castdermiro et al., 2014), affecting a wide range of cultivated species. It is associated with life-threatening sepsis and infections in various animals, including

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humans (Castdermiro et al., 2014). Vibriosis is one of the most important and the oldest recognized fish disease in marine aquaculture worldwide (Gonzalez et al., 2003). The main feature of this bacterial group is their capability to cause serious alimentary intoxication associated with the consumption of raw or undercooked contaminated fish or shellfish, thus posing a considerable public health threat. Pathogenicity, frequency of relapses and their severity, determined the choice of these four species as targets. 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 and it may be a mean to encourage aquaculture companies to diversify their production.

In the past, individual PCR assays have been developed for detection and identification of these fish pathogens, resulting in a relatively costly and time-consuming process, consequently there has been much interest in the development of multiplex PCR (m-PCR) method, thus reducing cost, time and effort. Although simultaneous detection of several pathogens with a multiplex PCR (m-PCR) 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 (Castdermiro et al., 2014), and reports of applications of these techniques on a routine basis in diagnostic laboratories are few (Chatterjee and Haldar, 2012). Several attempts to develop methods for the rapid and accurate diagnosis of edwardsiellosis have been made, including PCR-based methods (Castdermiro 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 (Castdermiro et al., 2014). In 2006, Fukui and Sawabe (2007) modified the method developed by Bramha Chari and Dubey (2006) and developed a one-step colony PCR targeting the 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 toxR 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. (2010), using a multiplex PCR tool. The main drawback of molecular methodology is that it does not discriminate between DNA from alive and dead microorganisms (Espiñeira et al., 2010). Success of this method depends on the selection of target genes, which should be species-specific, widely distributed and also stable in the genome (Chatterjee and Haldar, 2012). Considering the damages that some bacteria can cause to humans and fish, it is necessary to have a fast, simple, simultaneous (detection of different pathogens in one single reaction at the same time) and low cost detection method, being the primary objective of this study.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Four bacterial pathogen species were used in this study: *Edwardsiella tarda* (ACC 36.1), *Vibrio harveyi* (DSM 19023), *Vibrio anguillarum* (AQV 55.1) and *Vibrio alginolyticus* (CECT 521). The strains *Edwardsiella tarda* (ACC 36.1) and *Vibrio anguillarum* (AQV 55.1) used in the present study, virulent for teleost fishes from aquaculture, were kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain). Bacteria were cultured on Tryptone Soya Broth (TSB; Himedia), supplemented with 1.5% of sodium chloride (NaCl; Panreac), except for *E. tarda* that was cultured with 1% sodium chloride.

2.2 DNA extraction and PCR conditions

Bacterial DNA extraction was made with the NZY Tissue gDNA isolation kit (Nzytech, genes & enzymes) as indicated in the kit instructions. Bacterial DNA was quantified with a Nanodrop 2000 Spectrophotometer (Thermo scientific) in $ng\,\mu l^{-1}$. Individual PCR reactions were performed with the NZYTaq $2\times$ Colourless Master Mix, with 2.5 mM $MgCl_2$, 0.125 μM of each primer and template DNA (64.6 ng μl^{-1} for *E. tarda*; 89.5 ng μl^{-1} for *V. alginolyticus*; 110.8 ng μl^{-1} for *V. anguillarum*; 24 ng μl^{-1} for *V. harveyi*). The PCR reaction was initiated with a denaturation of 5 min at 95 °C, followed by 45 cycles of denaturation for 30 s at 95 °C, annealing 30 s at 51 °C and extension for 1 min at 72 °C. The final extension step consisted of 10 min at 72 °C. Amplified products were separated on agarose gel at 1.5% (w/v) with $1\times$ TAE buffer and visualized in UV transilluminator. The horizontal electrophoresis ran for 60 min at a voltage of 70 V. Primers used in this study are listed in Table 1 with respective target bacteria, name, forward and reverse sequences, melting temperatures (°C) and expected size band (bp). 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).

2.3 m-PCR analysis

Multiplex-PCR reactions were tested using the NZYTaq $2\times$ Colourless Master Mix, separate $MgCl_2$ (MB04902), with variable annealing temperatures (49 °C, 51 °C, 53 °C and 55 °C) and 0.125 μM of each primer. The PCR reactions were initiated with a denaturation of 5 min at 95 °C, followed by 45 cycles of denaturation for 30 s at 95 °C, annealing 30 s at the indicated temperature and extension for 1 min at 72 °C. The final extension step consisted of 10 min at 72 °C. m-PCR reactions were also made 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, and visualized in UV transilluminator. 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^{-1} for *E. tarda*; 89.5 μg μl^{-1} for *V. alginolyticus*; 110.8 μg μl^{-1} for *V. anguillarum* and 24 μg μl^{-1} for *V. harveyi*. Amplifications conditions were the same used for specificity testing.

Table 1. Characteristics of the primers used in this study and their references.

Target bacteria	Primer name	Sequences (5′–3′)	Melting temperature (°C)	Expected size (bp)	References
E. tarda	EtdF	AGCGCAGCTAACGGTAAAGT	57	426	This study
	EtfA_R	TGTAACCGTGTTGGCGTAAG	55		Sakai et al. (2007)
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		Ransangan and Lal (2013)
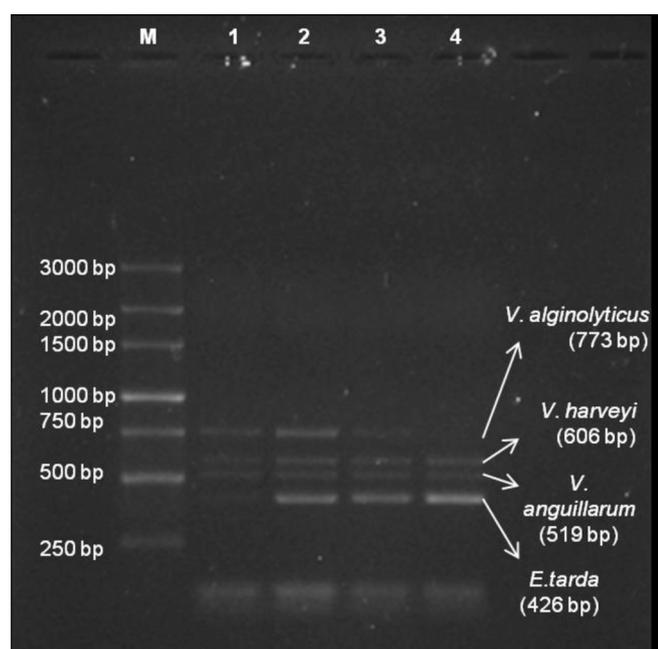
Fig. 1. Multiplex PCR at different annealing temperatures. M: 250 bp ladder; lane 1:49 °C; lane 2: 51 °C; lane 3: 53 °C; lane 4: 55 °C.

3 Results

3.1 m-PCR analysis

Four different annealing temperatures (49 °C, 51 °C, 53 °C and 55 °C) were tested and we obtained four bands with the expected size (*E. tarda* − 426 bp; *V. anguillarum* − 519 bp; *V. harveyi* − 606 bp; *V. alginolyticus* − 773 bp) at 49 °C, 51 °C and 53 °C and only three bands at 55 °C (*E. tarda* − 426 bp; *V. anguillarum* − 519 bp; *V. harveyi* − 606 bp). 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* (Fig. 1).

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 results obtained were the 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

4–6 mM of $MgCl_2$ is better to distinguish the bands of the four different pathogens: *E. tarda*, *V. anguillarum*, *V. harveyi* and *V. alginolyticus* (Fig. 2).

3.2 Sensitivity of m-PCR assay

Primers were 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 could be identified on agarose gels with clearness and without overlapping sizes (Fig. 3). The detection limit of the multiplex PCR amplification was as low as $0.4 \, ng \, \mu l^{-1}$ for *V. anguillarum*; $0.5 \, ng \, \mu l^{-1}$ for *E. tarda*; $1.5 \, ng \, \mu l^{-1}$ for *V. harveyi* and $5.6 \, ng \, \mu l^{-1}$ for *V. alginolyticus* (Fig. 4).

4 Discussion

Currently, aquaculture is one of the fastest growing food production systems in the world (Castdermiro et al., 2014). Disease management and assessment of cultured aquatic animals is a major concern in commercial aquaculture (Shi et al., 2012) and the molecular methods like PCR and multiplex-PCR are always more reliable to overcome pathology problems, being the last one more efficient due to multiple detection of pathogens in an only reaction. Multiplex-PCR 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; Castdermiro 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) and in the present work, several reaction conditions were tested for the development of this tool, including varying annealing temperatures (between 49 °C 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 pathogens tested (*E. tarda*, *V. alginolyticus*, *V. anguillarum* and *V. harveyi*) (Fig. 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
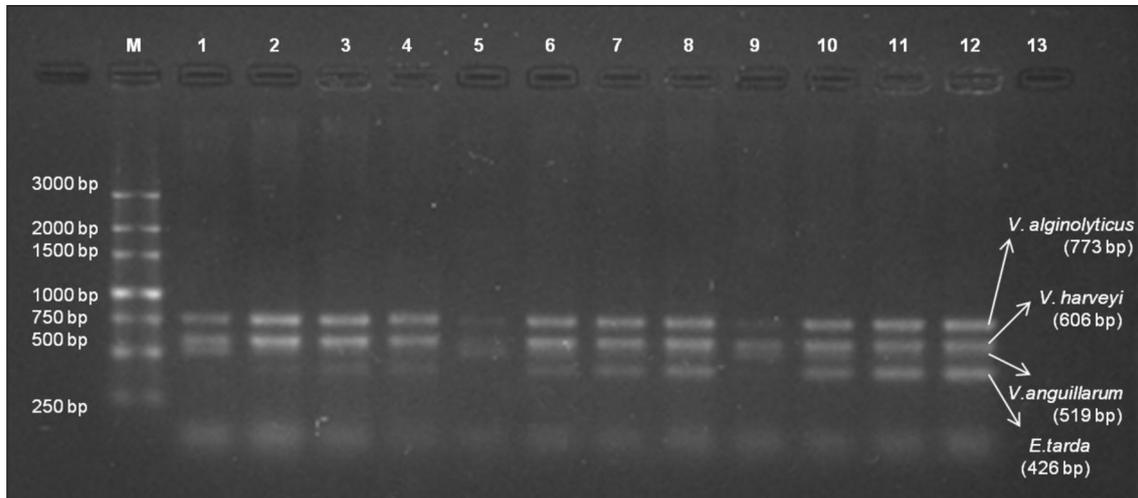
Fig. 2. Multiplex-PCR at different annealing temperatures and different concentrations of $MgCl_2$. Lane 1 – 250 bp ladder; lanes 2–5: 49 °C; lanes 6–9: 51 °C; lanes 10–13: 53 °C; lanes 2, 6, 10: 2 mM $MgCl_2$; lanes 3, 7, 11: 4 mM $MgCl_2$; lanes 4, 8, 12: 6 mM $MgCl_2$; lanes 5, 9, 13: 8 mM $MgCl_2$.
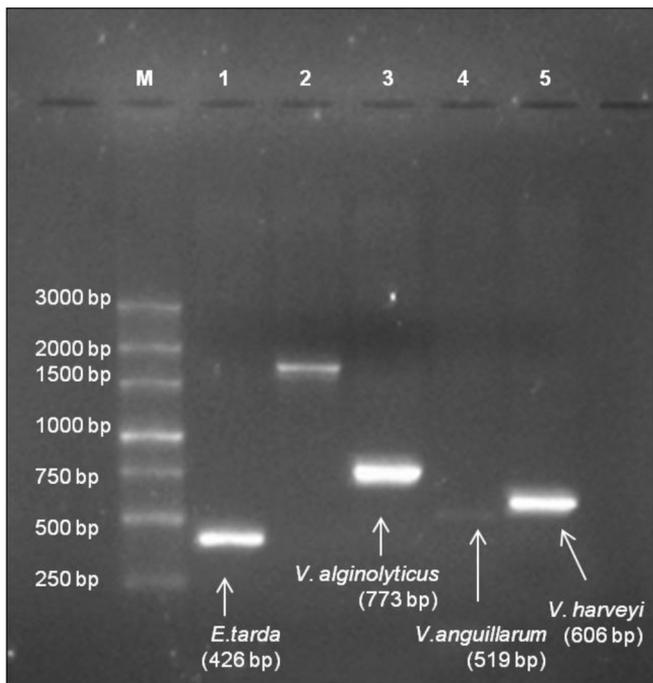

Fig. 3. Amplification of assay primers in monoplex reaction. M – 250 bp ladder; lane 1 – *E. tarda* (369 bp); lane 3 – *V. alginolyticus* (773 bp); lane 4 – *V. anguillarum* (519 bp); lane 5 – *V. harveyi* (606 bp).

specific under the conditions assayed, with only the specific target species showing amplification in the multiplex reaction (Fig. 3) and presented a satisfactory detection limit (Fig. 4). 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 (Castdermiro et al., 2014). Therefore, precise and early detection of pathogens in fish is essential for effective disease control (Altinok, 2011). Even though, and according to

Lievens et al. (2011), until 2011, it has not been reported the utility of the system for sensitive detection from complex samples, such as infected tissue. The results indicate that the multiplex PCR tool is suitable for detection of the four major pathogens tested (*E. tarda*, *V. harveyi*, *V. anguillarum* and *V. alginolyticus*). This method allows a diagnosis of edwardsiellosis and vibriosis in one working day; 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. 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 1. The PCR protocol described here has practical application for the rapid and early diagnosis of edwardsiellosis and different types of vibriosis. This PCR protocol could potentially be used to monitor these diseases in species of fish form aquaculture.

5 Conclusion

Although the molecular methods such as PCR and multiplex-PCR can bring multiple advantages, they may vary in amplification efficiency. In a multiplex PCR assay, and due to differences in primer length, nucleotide content and secondary structure, a target in high concentration might outcompete one or more of the other primers. Consequently, the detection limits for the targets at lower concentrations might become affected (Altinok, 2011). In general, 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 work carried out allowed us to establish the primer sequences and PCR multiplexing conditions, targeting 4 bacterial pathogens, which are currently the subject of strong concerns in the commercial aquaculture
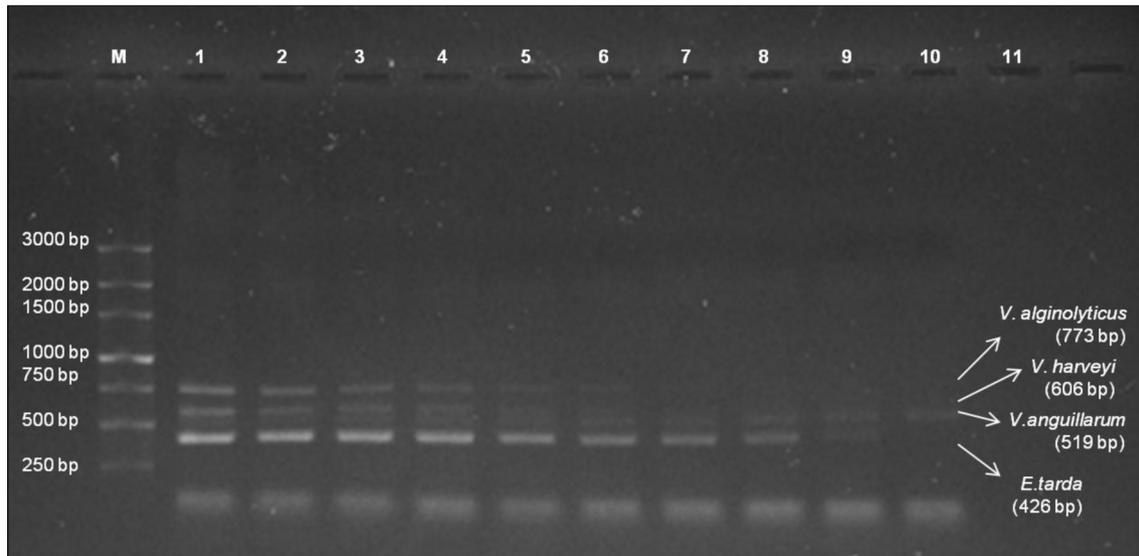
Fig. 4. Multiplex-PCR sensitivity test. Lane 1 – 250 bp ladder; lane 2 – mixture of different DNA (64.6 ng µl^{-1} for *E. tarda*; 89.5 ng µl^{-1} for *V. alginolyticus*; 110.8 ng µl^{-1} for *V. anguillarum*; 24 ng µl^{-1} for *V. harveyi*); lane 3 – mixture of different DNA (32.3 ng µl^{-1} for *E. tarda*; 44.8 ng µl^{-1} for *V. alginolyticus*; 55.4 ng µl^{-1} for *V. anguillarum*; 12 ng µl^{-1} for *V. harveyi*); lane 4 – mixture of different DNA (16.2 ng µl^{-1} for *E. tarda*; 22.4 ng µl^{-1} for *V. alginolyticus*; 27.7 ng µl^{-1} for *V. anguillarum*; 6 ng µl^{-1} for *V. harveyi*); lane 5 – mixture of different DNA (8.1 ng µl^{-1} for *E. tarda*; 11.2 ng µl^{-1} for *V. alginolyticus*; 13.9 ng µl^{-1} for *V. anguillarum*; 3 ng µl^{-1} for *V. harveyi*); lane 6 – mixture of different DNA (4 ng µl^{-1} for *E. tarda*; 5.6 ng µl^{-1} for *V. alginolyticus*; 6.9 ng µl^{-1} for *V. anguillarum*; 1.5 ng µl^{-1} for *V. harveyi*); lane 7 – mixture of different DNA (2 ng µl^{-1} for *E. tarda*; 2.8 ng µl^{-1} for *V. alginolyticus*; 3.5 ng µl^{-1} for *V. anguillarum*; 0.8 ng µl^{-1} for *V. harveyi*); lane 8 – mixture of different DNA (1 ng µl^{-1} for *E. tarda*; 1.4 ng µl^{-1} for *V. alginolyticus*; 1.7 ng µl^{-1} for *V. anguillarum*; 0.4 ng µl^{-1} for *V. harveyi*); lane 9 – mixture of different DNA (0.5 ng µl^{-1} for *E. tarda*; 0.7 ng µl^{-1} for *V. alginolyticus*; 0.9 ng µl^{-1} for *V. anguillarum*; 0.2 ng µl^{-1} for *V. harveyi*); lane 10 – mixture of different DNA (0.3 ng µl^{-1} for *E. tarda*; 0.3 ng µl^{-1} for *V. alginolyticus*; 0.4 ng µl^{-1} for *V. anguillarum*; 0.1 ng µl^{-1} for *V. harveyi*); lane 11 – control of the reaction, without DNA.

sector. These species can cause extensive damage and high losses and their early and rigorous detection is of fundamental importance. In future studies this tool must be improved through quantification of the sensitivity of the tool for blood samples and by testing it in a set of samples from farmed fish species.

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