Research Article

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Potential of PCR-based Molecular Diagnostic Methods for Rapid Detection of Bacterial Pathogens Causing Economically Important Diseases in Fishes

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Abstract: The intensive development of aquaculture during the last decades is related to increased spread of diseases caused by bacterial pathogens. They cause high mortality and significant economic losses. Molecular techniques are the best alternative to traditional methods offering fast, accurate and reliable methods for detection and identification of key pathogens. In this study we tested different PCR based methods for detection of the most common gram-negative pathogenic bacteria: *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida*. The q-RT-PCR is more sensitive, more accurate and faster than other PCR methods. It is however the most expensive one. A multiplex PCR (m-PCR) method for detection of the three important fish pathogens offers increased efficiency and reduces costs, time and labour. Therefore the m-PCR can be used for accurate and rapid identification of these bacteria..

Key words: Multiplex PCR, 16S rRNA*, Aeromonas salmonicida* subsp. *salmonicida*, *Flavobacterium psychrophilum*, *Yersinia ruckeri*

Introduction

Aquacultures are the fastest growing sector of food production worldwide, providing half of the fishes needed for human consumption and has a significant potential for further growth. The intensive development of aquacultures however created also conditions for rapid spreading of diseases caused by bacterial pathogens. They have a direct impact on production and lead to severe economic losses associated with high mortality or significant external lesions, ulcers, necrosis that make fish unsellable. Classic methods often require visible symptoms and significant time for diagnosis. Faster more accurate and reliable methods for detection and identification of key pathogens during the initial stages of diseases outbreaks are needed.

Molecular biology can offer new techniques as the best alternative to traditional methods. Using molecular tools pathogens can be detected at very early stages of outbreaks, infected fishes can be isolated and spreading of diseases can be prevented. Thus, antibiotic treatment may be extended to a limited number of fishes and thereby reduce treatment costs, production losses and the likelihood for appearance of antibiotic resistant bacteria (Wiklund 2000).

In this study, we tested PCR-based methods on the most common gram-negative pathogenic bacteria: *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida.*

Flavobacterium psychrophilum is a gram negative agent, causing cold water disease affecting

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mainly salmonids. Although its cultivation from fish tissues is performed on agar media, correct identification based on phenotypic characteristics is reported to be difficult and time consuming (Baliarda *et al.* 2002, Wiklund *et al.* 2000). Enteric red mouth disease caused by *Y. ruckeri* is a septicemic disease. Clinically healthy fish may be carriers of this pathogen and transmit it to other fish (Loghothetis *et al.* 1996). *A. salmonicida* causes furunculosis, a disease characterized by muscle lesions and associated skin ulcers and septicaemia. *A. salmonicida* is commonly isolated from both salmonids and non-salmonids (Gustafson 1992).

It is obvious that a more cost effective, sensitive and specific system is required for detection of these fish pathogens. Such system would also be useful for surveillance and monitoring fish populations (Altinok *et al.* 2008, 2003).

In this work we aimed to optimize the PCR assay for detection of *F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*.

Materials and Methods

Three types of materials were tested:

1) Isolates of pathogenic bacteria Flavobacterium psychrophilum, Yersinia ruckeri and Aeromonas salmonicida;

2) Frozen tissues of infected fishes;

3) 60 fish samples without external lesions, ulcers and necrosis were collected from fishery farms near Plovdiv.

Primers design: All nucleotide sequences used to design primes were derived from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). For *Aeromonas salmonicida* gene for 16S rRNA we used accessions AB027005; AB027006; HG938306; LT160807; LT160780. For *Flavobacterium psychrophilum* 16S rRNA gene accessions CP007207; CP007627; CP012586 and for *Yersinia ruckeri* 16S rRNA gene accession FN668391; FN668390; FN668389; FN668388; FN668386; FN668385. The multiple alignments of sequences were performed using Vector NTI 10.1 software (Invitrogen) and consensus sequence was used to design several sets of primers specific to *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida* and respectively suitable for normal, multiplex and qReal time RCR. Primers published in the literature were tested as well. Primers were ordered from Metabion AG. Martinsried, Germany, and upon arrival were dissolved in DNase-free water to 100 mmol.L^{-1} stocks. Before use 10 mmol.L^{-1} aliquots were prepared.

DNA isolation: Pathogens' DNA was isolated with QIAamp UCP Pathogen Mini Kit according to the instructions provided by manufacturer. Nucleic acid was quantified using spectrophotometer. When fish tissues were used they were first macerated with 10 µl of buffer containing 10 mM Tris HCl, 100 mM EDTA, pH 7.6.

Quantitative real-time PCR (qRT-PCR): Primers for the qRT-PCR analysis were designed to amplify between 120 and 140 bp fragments of 16S rRNA genes as described above. They were tested for detection of the three major fish pathogens, *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri*. Reactions contained 10 mL of SYBR Green Master Mix (Applied Biosystems), different dilutions of purified pathogens DNA and 200 nmol.L^{-1} of each gene-specific primer in a final volume of 20 mL. The qRT-PCR programme included: 50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 s and 60 C for 1 min.

Multiplex PCR: The primers used for each microorganism and the expected size of PCR products are presented in Table 1.

Reaction mixtures for both colony and multiplex PCR reactions were prepared in 200 µL PCR tube containing with 1 µL of each primer (10 mmol.L^{-1} concentration), 25 µL PCR master mix (Fermentas, Cat N K0171) and DNase-free water (supplied with the master mix kit) was added up to 50 µL final volume.

Initially the primers were tested separately by colony PCR. For this purpose small amounts of

Table 1. Best primer combinations suitable for simultaneous detection by multiplex PCR of *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida.*

Strain	Primers	Product size
F. psychrophilum	FP Fw2 5'-CAAGCGTTATCCGGAATCAT-3' FP3 Rev2 5'-CCGGTACGGCTACCTTGTTA-3'	~ 960 bp
Yersinia ruckeri	Y Fw2 5'-CACTTTCAGCGAGGAGGAAG-3' Y Rev2 5'-TCTGCCAAGTTCTGTGGATG-3'	~ 580 bp
A. salmonicida	AS Fw1 5'-CAGAAGAAGCACCGGCTAAC-3' AS Rev3 5'-ACAGCCTCCAAATCGACATC-3'	~ 323 bp

bacteria were picked-up from on colonies grown on Petri dishes with selective media with sterile tooth pick. Without the step of DNA extraction, bacteria were dispersed in PCR 50 µl reaction mixtures.

For multiplex PCRs 120 ng of extracted DNA was also added. Negative control consisted of 1µl (10 pmol/µL) of each primer 25 µl PCR master mix and ultra-pure water added up to 50 µL.

The PCR tubes were places TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried-out by using the following programs: For colony PCR initial incubation for 10 min at 94°C was used to breakup bacterial cells. For all other reactions initial denaturation at 94°C/3 min then 30 cycles of 94°C/45 sec; 60°C/45 sec; 72°C/45 sec, and a final extension step at 72°C/3min. PCR products were separated by agarose gel electrophoresis. For this purpose each sample was mixed with 5 mL of loading dye (Fermentas, Cat № R0611), loaded onto 1% agarose gel containing 0.5 mg/mL ethidium bromide (final concentration) covered with 0,5 X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with 1 kB DNA ladder (Fermentas GeneRuler Cat № SM0311). The PCR products were visualized by UV light and documented by BIO-VISION+3026. WL system (Vilber Lourmat).

Results and Discussion

Several experiments were performed using isolated DNA from *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida* to determine the primers specificity and for optimization of PCR conditions. The q-RT-PCR did allow us to detect and quantify the pathogens even in dilutions down to 1:1000, which prove that the method is more sensitive, more accurate and faster than other PCR methods in laboratory conditions and with well purified DNA samples. It is known however that various substances, such as haemoglobin, bacterial components and high concentrations of DNA can inhibit the amplification and can affect the results of the diagnostic tests (Wilson 1997). q-RT-PCR is using also quite expensive reagents. When we need to analyze between several hundred and a few thousand fish samples, such method appears to be far too costly.

Colony PCR was used also to test primers for the multiplex PCR. Both single isolated stains and mixtures of the three pathogens *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida* were used. The results are presented on figure 1. Panel of the figure represents product amplified from individual pathogen species, while panel B

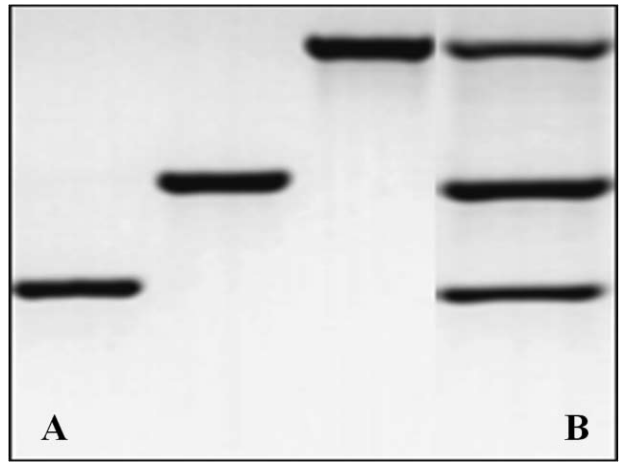

Fig. 1. PCR amplification products of fragments 16 S rRNA. Panel **A** presents products obtained by colony PCR of pure cultures of fish pathogens. From left to right *Aeromonas salmonicida*, *Yersinia ruckeri* and *Flavobacterium psychrophilum*. Panel **B** represent products of multiplex PCR performed with mixture of all three pathogens. Products were separated on 1% agarose gel

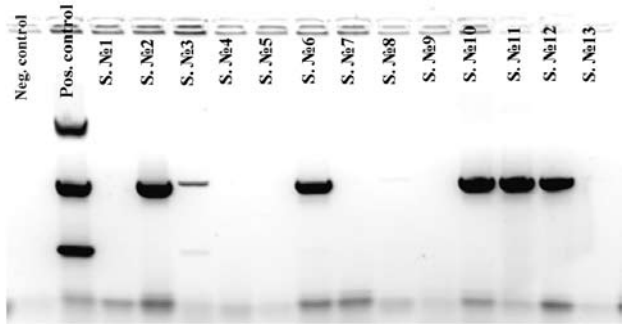

Fig. 2. Multiplex PCR performed isolates from tissues of 6 infected and 7 healthy fishes. Products were separated on 1% agarose gel

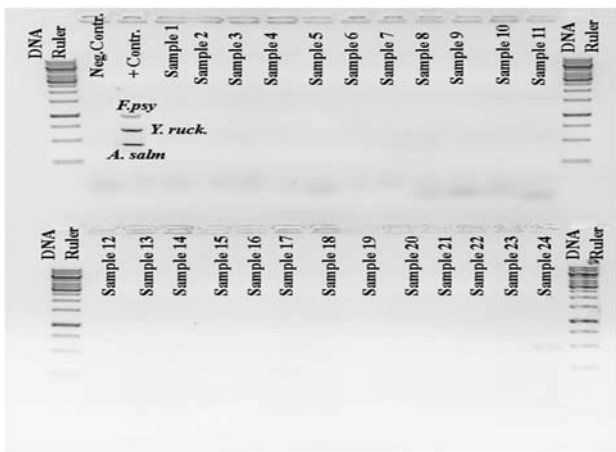

Fig. 3. Multiplex PCR performed isolates from tissues of fish samples without external lesions, ulcers, necrosis, collected from fishery farms near Plovdiv. Products were separated on 1% agarose gel

products amplified in mixed samples (Fig. 1A, B).

Next we used isolated DNA from 7 healthy and 6 infected fish tissues by multiplex PCR. Five of the infected samples were undoubtedly identified. One sample - №3 gave a weak signal (Fig. 2). Healthy samples did not produce any artificial signals.

Finally isolates from the 60 fish samples were next tested by multiplex PCR and the same primers – none of the fish were infected (Fig. 3).

The multiplex PCR method offers increased efficiency and reduces costs, time and labour when for detection of the three important fish pathogens are needed. Our conclusions are in agreement with other authors. E. g., Altinok *et al*. (2008) developed of multiplex PCR assay for simultaneous detection of five bacterial fish pathogens.

Conclusions

In conclusion, the results of this research are still preliminary but they have demonstrated the power of the multiplex PCR assay as molecular diagnostic method for bacterial pathogens detection in fish farms. We expect to expend this research during the spring of 2017 because the cold-water aquaculture species are susceptible to diseases which generally appear in per acute and acute forms in young fish during spring months (Horne & Barnes 1999). We expect also to extend study to other bacterial pathogens following the protocols for detection and identification of pathogenic bacteria used by researchers in other countries (Altinok *et al.* 2008, 2003, LeJeune *et al.* 2000, Urdaci 1998, Rys & Persing 1993).

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