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Development of diagnostic method for marine fish pathogens

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Abstract
Rapid detection of bacterial fish pathogens in aquaculture is very important for effective disease control in Turkey which has total production capacity of 240 thousand tons of cultured fish. Conventional microbiological methods are time consuming and lack sensitivities to detect latent marine pathogens. The application of new biotechnologic methods for detection will make a significant impact on the development of such rapid diagnostic approach to aquaculture.
In this study, we have used gene based PCR methods to developed rapid diagnostic kit for fast detection of bacterial infection in marine fish. Four different modified oligonucleotid primers (vvhA, aero, gyrB and hsp70) have designed to detect of *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Mycobacteria* spp. from marine fish. As a result of this study, four different bacterial agents *Aeromonas salmonicida*, *Vibrio anguillarum*, *Pseudomonas anguilliseptica* and *Mycobacterium marinum* simultaneously has been identified and detected with using 8 primers in same-single tube in very short time.

Keywords: Fish diseases, PCR

1. Introduction
In recent years, aquaculture production in Turkey is developing rapidly with the addition of the dams, lakes and ponds established in cage systems as well as farms and the number of them has reached to 4600 [1]. Growing the desired level of commercial production and contributing to the country economy depend on factors such as culture on suitable conditions, prevent, protection and fast detection from diseases.
The most commonly observed diseases in marine fish are furunculosis caused by *A. salmonicida*, vibriosis caused by *Vibrio* spp., *Pseudomonas* infection by *Pseudomonas* spp. and mycobacteriosis *Mycobacteria* spp [2, 3]. These diseasase causes economically devastating losses in cultivated salmonids and non-salmonid fish in fresh and marine waters [4, 5].
Microbiological, biochemical, immunological and histopathological methods were used for identification and determination of the causative agents of fish disease in aquaculture in the past [4, 6]. These time-consuming and highly labor-demanding methods are based on the laboratory transfer of sample and microbial cultivation of bacteria for several weeks and then biochemical tests and the phenotypic diagnosis [2, 7]. Rapid isolation, identification and detection of causative pathogens through sensitive and strain specific methods are required and essential for controling bacterial diseases in aquaculture. The amplification of molecular genetic techiques can now use for detection disease agents in suspected fish samples. These alternative molecular approaches such as PCR, based on virulence genes or 16Sr DNA genes are used for identification of fish pathogens [8, 9, 10, 11].
In this study, we focused developing a practical diagnostic kit for four bacterial agents concern for marine fish in Turkey. We selected bacterium-specific, virulence related genes for multiplex PCR amplification to permit more bacterial detection. Selected genes were the cytolisin gene (vvhA) which is present in *V. anguillarum*, aerolysin gene (aero) related to hemolytic, adhesion properties and mucinase production of *A. salmonicida*, DNA gyrase gene (gyrB) that encode the subunit B protein of topoisomerase type II as targets for *P. anguilliseptica*, heat shock protein gene (hsp70) which is the putative virulence genes of *M. marinum*. Although, PCR has been applied to the detection of multiple bacterial pathogens in aquaculture, this report is the first and preliminary results of devoloping diagnostic kits for four type of marine fish pathogens in one tube/3 hours simultaneously.

2. Materials and Methods

2.1. Bacteria Culture Conditions

The bacteria used in this work were *A. salmonicida* ATCC 33658 were cultured on tryptone yeast extract agar (BBL, USA) at 22 to 25°C and 24 to 48 hrs., *M. marinum* ATCC 927 were cultivated on Middlebrook 7H11 medium (BBL, USA) at 22 to 25°C for 5 to 10 days, *V. anguillarum* and *P. anguilliseptica* from our isolates were incubated at 22 to 25 ^{o}C for 2-4 days in trypticase soy broth (BBL, USA) [2].

2.2. Bacterial DNA Extraction

All tested bacteria were grown in broth at optimum conditions. Cells from overnight cultures were washed and resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA (Amplichem, Germany). Chromosomal bacterial DNAs of were extracted using Qiagen miniprep DNA extraction kit based on manufacturer's instruction (Qiagen, USA).

2.3. Primers

The primers used in this work were *A. salmonicida* (424 bp) (AeroF: AGCGGCAAAGCCCGTCTATCCA; AeroR: AGTTGGTGGCGGTGTCGTAGCG), *V. anguillarum* (520 bp) (vvhAF: CCGCGGTACAGGTTGGCGC; vvhAR: GGCCACCCACTTTCGGGCC), *P. anguilliseptica* (200 bp) (gyrBF: GCTGTCCGAAGAACTTGTACTGAC; gyrBR: ATTCTTGAAGGTGTCAGCCGATGG), *M. marinum* (580 bp) (hsp70F: CGAAGACTCAATGGCTGATCTT; hsp70R: TTGCTCAATTTTGCATCACGTAA) respectively.

2.4. Multiplex PCR Amplification

Multiplex PCR mixtures (50 µl volume) each contained 50 to 100 ng of purified genomic DNA, 200 µM each deoxynucleoside triphosphate (Fermantas, Germany), 400 nM each primer (Metabion, Germany), 2.5 mM $MgCl_2$, 1x reaction buffer, and 2U of Taq polymerase (Thermo, USA). Thermal cycling was performed with a Teche (Germany) and included an initial incubation at 95°C for 3min followed by 30 amplification cycles. Cycling included denaturation for 30 s at 95°C followed by annealing for 1 min at 52, 54, 56, 58, 60, or 62°C. Extension was for 45 s at 72°C, and cycling was concluded with a final elongation for 5 min at 72°C. All multiplex products were checked by electrophoresis on 1% agarose (Thermo, Germany) gels and stained with 0.5 µgml $^{-1}$ ethidium bromide (Applichem, Germany).

3. Results and Discussion

In this research, *A. salmonicida* ATCC 33658, *M. marinum* ATCC 927, *V. anguillarum* and *P. anguilliseptica* were cultured in TSB medium and their DNAs were extracted. Eight oligonucleotide primers were designed and used to simultaneously detect four different marine fish pathogens by mPCR in a single tube. They are targeted at a species-specific and virulence regions of the *A. salmonicida* aero gene, *M. marinum* hsp70 gene, *V. anguillarum* vvhA gene and *P. anguilliseptica* gyrB gene. A number of key parameters of mPCR like anneling temperature and time, primer concentration, Mg^{+2} concentration, polimerase quantity were optimised for each gene amplifications. The mPCR results demonstrated that mixture genomic DNAs of four bacterial pathogens were effectively amplified to identify bacteria belonging to *A. salmonicida*, *V. anguillarum*, *P. anguilliseptica* and *M. marinum*. Size of mPCR products in agarose gels were 424 bp, 580 bp, 520 bp, 200 bp respectively (Fig.1.). Eventhough, primer nucleotid sequences that we used had some modifications, our PCR results were supported by Izumi and Suzuki [12], Gonzalez *et al.* [13], and Tsai *et al.* [14]. Aerolysin and hemolysin genes which are related to putative virulence genes of *Aeromonas* spp. were used to detect *A. salmonicida* ATCC 33658 in our assay. Yousr *et al.* [15] has reported PCR data of same gene for *Aeromonas* spp. in samples of shrimp and peeled shrimp taken from environmental sources. Moreover, vvhA gene of *V. anguillarum* were detected as similar to size (519 bp) in single PCR to the report of Sanjuan and Amora [16]. The results of *Pseudomonas* positive amplicons achived with size of 200 bp using gyrB gene as described in Lopez *et al.* [17].

Bacterial diseases considered the most serious disease problem among all fish populations resulting in rapid and massive mortalities. Bacterial pathogens create problems when stress conditions increase under intensive culturing and bad environmental parameters. In this case, healthy looking fish can carry some pathogens and create a serious risk for spread of congious diseases in aquculture. Since most of the time, prevalence of bacterial diseases may change depending on time of year, water temperature and type of bacterial agents [17]. Therefore, the early diagnosis of aquatic infection is essential for disease management and fish welfare in aquaculture worldwide [18].

A multiplex PCR method that amplified target genes for each bacterium has been developed for *A. salmonicida*, *M. marinum*, *V. anguillarum* and *P. anguilliseptica* in a single reaction in shorter period of time (3 to 4 hours) succesfully. Amplification results also confirmed that slow growing, low numbered and non-culturable viable bacteria can be detected without bacterial culturing process. This assay appeared to be very sensitive, with low detection limit of 5 CFU of *A. salmonicida*, 6 CFU of *M. marinum*, 4 CFU of *V. anguillarum* and 8 CFU of *P. anguilliseptica* in pure culture. Gibello *et al.* [19] and Byers *et al.* [20] have stated the similar detection limits of PCR mixture of bacteria. In addition, this method has more advantages in terms of it specificity and ease of cost, compared to bacteriological and biochemical methods. The detection of multiple pathogens simultaneously in routine diseases monitoring procedures in aquaculture facilities will constitute a step further in the development of techniques for rapid, specific, cost reduced and effective diagnosis.

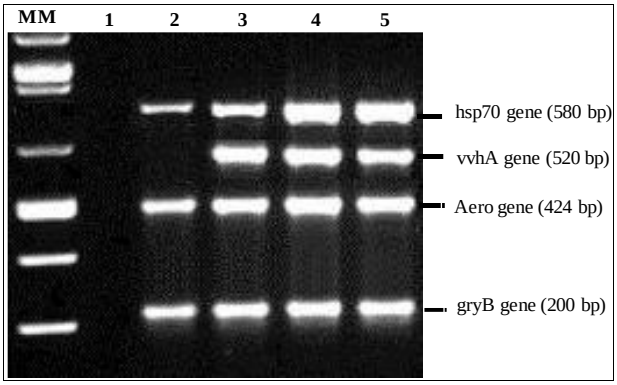

Fig 1: Detection of *A. salmonicida*, *M. marinum*, *V. anguillarum* and *P. anguilliseptica* by mPCR. Molecular Marker, 1.Negative control, 2, 3, 4, 5. mPCR amplicons of targetted genes.

4. Conclusion

There is growing interest in the diagnosis of main fish diseases in aquaculture. In conclusion, the mPCR method developed in this study could overcome some difficulties in tradiotional methods such as low densities and slow growth and time, and increase the sensitivity of the multiple detection. These results would be the first and preliminary results for developing high throughput and sensitive diagnostic kits of aquacultural diseases in the marine environment in future. Such methods may be further developed to include other important marine pathogens and extended to testing of other marine organisms (*e.g.* shellfish).

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