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Designing *streptococcus iniae* diagnostic kit by multiplex polymerase chain reaction method in Iranian rainbow trout

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Abstract

Streptococcus is a large genus of bacteria which cause Streptococcosis in aquatic animals. In Iranian fishes, *Streptococcus iniae* cause Streptococcosis. This disease was formerly diagnosed as golf ball disease. The current study aimed to produce a polymerase chain reaction (PCR) kit with five local suspicious internal controls with clinical symptoms such as melanosis, pallor, exophthalmia and bleeding in the anterior part of the eye and retina, fin base, vent, and gills through histopathologic technique to diagnose Streptococcosis using MultiPlex PCR method. In this study, two bands of 725 base pair and 340 bp indicating the host (control) and the pathogen bacteria were observed, the PCR was confirmed and the Streptococcosis in the suspicious samples was diagnosed. The aim of this study demonestrated designig new kit for diagnosed the *diagnose* Streptococcosis using to MultiPlex PCR method.

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Introduction

Today, the growing artificial propagation of fishes has raised new environmental issue and has increased the rate of infectious diseases and mortality among fish population. In this regard, Streptococcosis has great importance due to wide distribution, mass causality in fish population, and also the chance of transmission to human. Besides *Streptococcus* genus, there are some affiliated genera which cause similar diseases such as Lactococcus and Enterococcus; hence, any one of these diseases is considered as Streptococcosis. *Streptococcus* is a large genus of bacteria causing Streptococcosis in aquatic animals. There are different *Streptococcus* species in different regions which causeStreptococcosis in fish; for example, *S.difficilis* can be found in Syria, S. *milleri* in Japan, and *S.parauberis* can be isolated in North of Spain. According to the studies conducted in Iran, *Streptococcus* iniae is a pathogen in the Iranian fish-farms. The early symptoms of Streptococcosis contains anorexia, melanosis, pallor, and convulsive swimming; with disease progression, some other symptoms such as one and two side(s) exophthalmia, bleeding in the anterior part of the eye and retina, fin base, vent, and gills occur. The most important internal organs dealing with this disease are spleen, liver, brain, intestine, and heart. Streptococcosis may cause high rate of mortality (50%) among fishes within three to seven days (Roozbahani *et al.*, 2009).

In 1977, global economy estimated that the damage caused by *S.iniaie* infectionin the fish farming industry wasabout $100 million, and 1% of the amount was reported in the United States (Agnew *et al.*, 2007).

To diagnose Streptococcosis, different techniques have been recommended by researchers and scientists, which some of them are as follows: MultiPlex PCR, Restriction Fragment Length Polymorphism (RFLP), PCR simple and serological methods such as Enzyme-linked Immunosorbent Assay (ELISA), or traditional methods such as culturing or Gramm staining, which none of them can be verified; therefore, false-negative results are predicted. Hence, using host's tissue as an internalcontrol to differentiate false-negative results (caused by test errors) from true-negative ones can increase the sensitivity of PCR diagnostic.

The aim of this study The aim of this study demonestrated designig new kit for diagnosed the Streptococcosis using to MultiPlex PCR method.

Material and methods

The current study was conducted from August 2012 to August 2014 in Tehran, Iran. *Streptococcus* iniae species was purchased from the Iranian Veterinary Organization. The suspicious samples were also provided from a contaminated Rainbow Trout farm in Mazandaran Province, Iran by the authorization of the Iranian Veterinary Organization. Samples were stored in a 37% formalin solution and transferred to the laboratory.

DNA Extraction

Bacterial colonies were picked from culture and suspended in lysing buffer containing sucrose 320 mM, Tris 10 mM, $MgCl_2$ 5mM, and 1% SDS. The tube was kept in a termoblock apparatus at 50°C for one hour. Then, bacterial DNA was extracted by phenol chloroform method and precipitated by absolute ethanol. DNA of fish tissue was also extracted accordingto the directions of manufacturer (Bioneer, South Korea).

Primer and Polymerase Chain Reaction

To prevent false-negative results in PCR method, two pairs of primers, based on 16S rRNA of S.iniae and 18S rRNA *of Oncorhynchus mykiss* (Roozbahani *et al.*, 2009). , with the following sequences were applied in the multiplex PCR:

16S rRNA :

STRinF 5'-GGTAAGCCGTATCGGAAGGT-3'

STRinR 5'-CCTAGC-3'TCCTTGTCAATGGAG

18S rRNA:

Onmy F 5'-CTGTGGCAATTCTAGAGC-3'

Onmy R 5'-CGTCCCTCTTAATCATGG-3'

The final volume was adjusted to 23µl with 10µl 2X Ampliqon 2 Master Mix, 1µl S.iniaePrimerR/F,2µl

(40 pmol each) *O.mykiss* PrimerR/F,3µl (1µgr) template DNA, 7µl DW.

PCR final product using S.iniae 16S rRNA and 18S rRNA primers were 340 bp, which had significant difference. BioRad CFX96 Real-time cycler was used to study development.PCR was amplified under the following conditions: early denaturation at 94°C for 10 minutes in 30 cycles containing denaturation for 45 seconds, annealing at 53°C for 45 minutes, and extension at 72°C for one minute, and finally the reaction was completed at 72°C for five minutes.The gradient thermocycler used in this study.

Electrophoresis

After completing the reaction, 10µl of PCR product was electrophoresed on 1.5% gel agarose and illustrated by 250 nm wavelengths UV in UV DOC apparatus. The samples were loaded beside a 100bp marker.

After adjusting the system, at least five samples, which their contamination had been approved by histopathological methods, were tested by the MultiPlex PCR method.

Results

Considering the aforementioned MultiPlex PCR method, proliferation DNA segment of the host implied proper amplification and detection; in such a way that, observing two bands of the host and the pathogen indicated proper conduction of PCR and contamination of the suspected samples; the segments with 752 bp and 340 bpbelonged to the host and the pathogen, respectively (Figure 1). Observing only the host related band in the PCR product implied proper PCR conditions and showed that the sample was not infected with *S.iniae;* this result was definite, without common test errors (Figure 2). Observing two pairs of *S.iniae* and the fish designed primers simultaneously with the DNA extracted from the contaminated fish tissue as a template in MultiPlex PCR implied that PCR was conducted under improper conditions and common test errors. In the current study, the host and pathogen bands with 725 bp and 340 bp lengths were observed in all the five samples.

Table 1. Diagnostic Kits Designed from 2000 to 2009 for Fish Pathogens.

Virus	Bacteria	Parasite
IHNV (Overturf *et al.* 2001)	*Pseudomonas plecoglossida (*Sukenda *et al.* 2000)	*Myxoboluscerebralis*Kelley *et al.* 2004
WDSV (Getchell *et al.* 2002)	*Flavobacteriumpsychrophilum*(Sukenda *et al.* 2000)	*Ichthyophthiriusmultifiliis*(Jousson *et al.* 2005)
RSIV (Caipang *et al.* 2003)	*Edwardsiellaictaluri*(Bilodeau *et al.* 2003)	*Ceratamyxashasta*(Hallett *et al.* 2006)
AHNNV (Grove *et al.* 2003)	*Piskiricketssia**Rain**bow Troutis*(Corbeil *et al.* 2003)	*Lepeoptheirus*(McBeath *et al.* 2006)
LMBV (Goldberg *et al.* 2003)	*Renibacterium**Rain**bow Troutinarum*(Powell *et al.* 2005)	*Kudoathyristes*(Funk *et al.* 2007)
KHV (Gilad *et al.* 2004)	*Clostridium botulinum type E* (Getchell *et al.* 2006)	*Ovipleistophoraovariae*(Phelps *et al.* 2007)
ISAV (Munir *et al.* 2004)	*Aeromonas**Rain**bow Trouticida*(Balcaza *et al.* 2007)	*Henneguyaictaluri (Griffin et al. 2008)*
Betanodavirus (Dalla Valle *et al.* 2005)	*Flavobacteriumcolumnare*(Panangala *et al.* 2007)	*Nucleospora**Rain**bow Troutis*(Foltz *et al.* 2009)
Aquabirnavirus (Hirayama *et al.* 2005)	*Moritella viscose* (Grove *et al.* 2008)	*Parvicapsulaminibicornis*(True *et al.* 2009)
VHSV (Chico *et al.* 2006)		
CyHV-2 (Goodwin *et al.* 2006)		
Alphavirus (Graham *et al.* 2006)		
SPDV (Hodneland and Endresen 2006)		
Pilchard Herpesvirus (Crockford *et al.* 2008)		
SVCV (Yue *et al.* 2008)		

Discussion

Bacterial diagnosing methods are traditionally based on culturing techniques, which usually take several days. In the recent years, epidemiology of the disease and its outbreak in fish farmshas drawn the attentions toward PCR method and its high speed comparing microbiological culturing methods; in such a way that, conducted studies from 2000 to

2009 led to design diagnostic kits for 33 fish pathogens containing fifteen viruses, nine bacteria and nine parasites (Table 1) (Getchell, 2009).

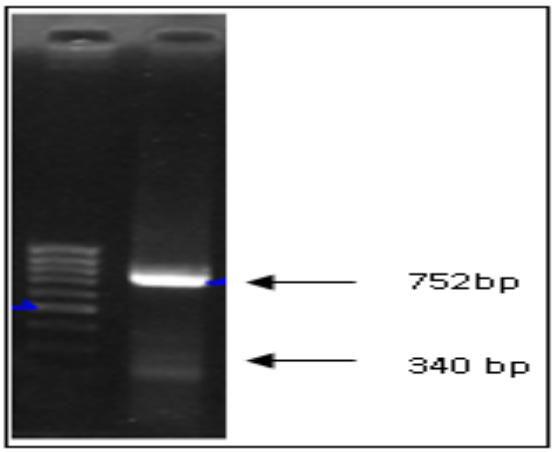

Fig. 1. PCR Product Electrophoresis on 1.5% Gel Agarose.In positive samples, the bands with 725 bp and 340 bp proliferated from Rainbow Troutand pathogen genes were observed beside a 100 bp marker.

Using internal control samples to diagnose some human pathogen microorganisms is practically exploited; in such a way that, using these internal control samples to increase the accuracy of diagnosing fish farming diseases still paves the early steps and has to be developed.

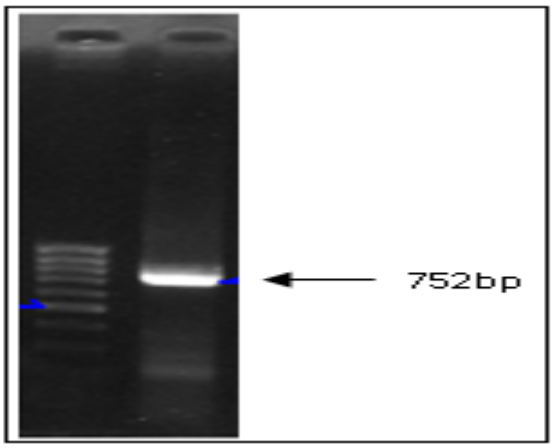

Fig. 2. PCR Product Electrophoresis on 1.5% Gel Agarose.In negative samples, only the band with 725 bp proliferated from fish gene was observed beside a 100 bp marker.

The study conducted in Thailand by Itsoro *et al.* (2012) has evaluated the contamination by *Streptococcus agalactiae, S. iniae,*and *Lactococcus garvieae*. In this study, to evaluate *S. iniae,*LCTO primer was used anda 870 bp band formed on gel electrophoresis. Diagnosing different pathogens simultaneously was the advantage of this study but due to lack of internal control amplification comparing to the designed kit in the current study, it had more errors (Itsaro, 2012).

Rodkhum *et al.* (2012) in Thailand conducteda similar study. They evaluated the contamination of fishes by *S. iniae* and *S.agalactiae*. In their study, Icto gene with 720 bp and 16S rRNA with 220 bp were selected as aiming genes in *S. iniae* and *S. agalactiae*, respectively. PCR product was electrophoresed on 1.5% gel agarose under UV radiation with 260 nm wavelength. Then, the results of Dulplex-PCR were compared with the PCR results for any one of these microorganisms with the same genes and the obtained results showed no difference between the results of the two microorganisms separately in Dulplex-PCR and PCR techniques, and the PCR products obtained bythe applied primers had no effect on each other (Rodkhum C., 2012). The study of Rodkhum *et al.* did not use internal control, which can be considered as an advantage of the current study.

The study conducted in Iran by Adel Haghighi *et al.* (2009) compared two histopathologic and MultiPlex-PCR methods to diagnose yersiniosis in Rainbow Trout. This study was conducted in 2009 in Science and Research branch of Islamic Azad University, Tehran, Iran. According to the conducted study, 20 suspicious samples with typical clinical symptoms such as acute septicemia in internal tissues and superficial bleeding around mouth and vent were evaluated by the mentioned methods. To prevent false-negative results in the PCR method, two pairs of primer based on 16S rRNA of *Yersinia ruckeri* and 18S rRNAof *Oncorhynchus mykiss* were used in the MultiPlex PCR. This study resulted in designing a PCR kit with higher sensitivity and specificity than the previous ones. Considering the point that Rainbow *Trout* tissue was used as the internal control in this study, the accuracy of PCR was approved (Pier

et al., 1976). Hence, this study was the most compatible Iranian one with the current study.

Considering the different studies conducted to diagnose *S. iniae* through PCR, the current research is unique in using Rainbow *Trout* tissue as the internal control. To design the kit, besides using the most preserved sequence of 16SrRNA of the pathogen as primer, to diagnose definite contamination of negative samples an internal control based on the host 18SrRNA was used. Negative or positive band of host gene should be proliferated in the case of contamination of negative samples to the pathogen. This process differentiates false-negative results (caused by test errors) from true-negative ones. It is obvious that PCR diagnostic kit designed in the current study was highly sensitive.

Considering the point that in MultiPlex PCR per run,one to ten primer(s) can be used simultaneously and also diagnosing three to four types of common Rainbow *Trout* diseases in MultiPlex PCR per run; in near future, the kits can be designed, which in addition to diagnosing multiple pathogens though host tissue as internal control, have higher sensitivity and accuracy comparedwith the older ones.

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