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Full Length Research Paper

Detection of *Streptococcus iniae* and *Lactococcus garvieae* by multiplex polymerase chain reaction (PCR) in some rainbow trout farms of Iran

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Streptococcosis is one of the most important bacterial diseases in farmed salmonid fishes. *Streptococcus iniae* and *Lactococcus garvieae* are known as the major pathogens of streptococcosis and lactococcosis in the rainbow trout, *Oncorhynchus mykiss*. The present study accomplished the detection of the two mentioned bacteria in some rainbow trout farms in the west of Iran. A total of 50 fish samples clinically suspected of having streptococcosis were collected from 10 rainbow trout farms. Samples of liver, kidney, spleen and heart were cultured on brain heart infusion agar medium. The bacterial isolates were identified using standard biochemical tests. Identification of the isolates was approved by multiplex PCR. *L. garvieae* and *S. iniae* were identified simultaneously in the fish tissues. The results of this study showed overcoming *L. garvieae* in isolated samples that indicated epidemiological importance of lactococcosis. Multiplex PCR was found as an effective tool for the rapid and specific detection of *L. garvieae* and *S. iniae* from fish tissues.

Key words: Streptococcosis, Lactococcosis, fish, Iran.

INTRODUCTION

The major pathogenic species of streptococcal infections are *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum* and *Carnobacterium piscicola* (Bercovier et al., 1997; Eldar et al., 1997). The first streptococcal infection in cultured fish was reported in rainbow trout (*Oncorhynchus mykiss*) in Japan (Hoshina et al., 1958). This fish is one of the most susceptible species in fresh water fishes. It was proven that the *L. garvieae* is one of the important Gram-positive coccus for cultured fish (Eldar et al., 1996). Streptococcosis can be treated by some antibiotics including erythromycin, florfenicol and amoxicillin (Treves-Brown, 2000; Yanong et al., 2005).

Currently, PCR reactions are exploiting the diversity of sequences of 16S rRNA genes in *L. garvieae* (Zlotkin et al., 1998). Different studies showed that streptococcal infections are not a single entity, but rather a complex of similar diseases caused by Gram positive cocci belonging to several genera, including Streptococci (Eldar et al., 1994) Lactococci (Williams et al., 1990), Vagococci (Collins et al., 1989) and Enterococci (Kusuda et al., 1991). Several vaccines have been developed against streptococcal bacteria (Eldar et al., 1997; Shelby et al., 2002). *S. iniae* was isolated from ornamental cyprinid fishes (Russo et al., 2006). Different genera and species of Gram-positive and catalase-negative cocci are pathogenic to fish (Gittino et al., 2003). Moreover, some molecular technique including taxonomic studies based

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Table1. Primer sequences used for PCR amplification and the expected amplicon sizes.

Primer pairs	Sequences (5´ to 3´)	Target gene	PCR amplicon (bp)	Pathogen
Lox-F Lox- R	(5´– AAGGGGAAATCGCAAGTGCC- 3´) (5´– ATATCTGATTGGGCCGTCTAA- 3´)	*lctO*	870	*S. iniae*
PLG- F PLG- R	(5´– CATAACAATGAGAATCGC- 3´) (5´– GCACCCTCGCGGGTTG- 3´)	16S rRNA	1,100	*L. garvieae*

on DNA-DNA hybridization studies and sequence analyses of 16S rRNA indicated that *Enterococcus seriolicida* (Kusuda et al., 1991) is in fact synonymous with *L. garvieae* and should be classified as *L. garvieae* (Eldar et al., 1996).

L. garvieae is an emerging zoonotic pathogen that has been isolated from various species of fish and humans (Mannion et al., 1990; Eldar et al., 1996). With the development of intensive aquaculture, streptococcal infections of fish have become a major problem worldwide (Kitao, 1993).

Outbreak of disease in rainbow trout farms of Iran were reported by Akhlaghi and Mahjor (2004) and Soltani et al. (2005). Culture of salmonids is one of the fastest growing industries in Iran in the recent years, and in this country majority of water resources have been allocated to rainbow trout farming. One of the most economical regions in rainbow trout production is located in Iran and increased production of these species infectious diseases such as Streptococcosis has occurred there too. Therefore, this study aimed at identifying the pathogens in fishes affected by Streptococcosis using Multiplex PCR method.

MATERIALS AND METHODS

Sampling and isolation of bacterium

During spring and winter 2009, sampling from 10 rainbow trout (*O. mykiss*) farms in Chaharmahal-va-Bakkhtiary province (located in West of Iran) was done. Infected or suspected fishes were randomly sampled from selected farms and then organs such as brain, kidney, spleen and liver sterile swabs were streaked on brain heart infusion agar plate (BHIA; Difco, USA) supplemented with 1.5% NaCl. Plates were transferred to the laboratory, where they were kept on ice.

Isolation of pathogens and biochemical analysis

Plates transferred to the laboratory were incubated at 25°C for 48 h for growing the colonies. Single colonies from plates with pure culture growth were re-streaked on the BHIA media to obtain pure isolates. In each of the grown colonies, catalase and Gram-staining tests were performed and Gram-positive and catalase negative cocci were sent for PCR Test. In each step of PCR testing, distilled water as negative control was used.

DNA extraction

Pure colonies were put in tubes with 100 µL distilled water. Then DNA was extracted according to kit of extracting DNA designed by CinnaGen Co, (DNPTM kit Cinnagen, Iran).

Primers

The target gene and oligonucleotide primer set used for the detection of each of the two fish bacterial pathogens in the m-PCR are indicated in Table 1. All primers were synthesized by CinnaGen Co. (Tehran, Iran).

PCR amplification test

A multiplex PCR that detects the genes encoding lactate oxidase-encoding (lctO) and 16S rRNA was performed using the primers described by Mata et al. (2004a). The PCR assay was carried out in a total volume of 50 µL of mixture containing PCR buffer 10x, 1.5 mM of $MgCl_2$, 250 µM of each of deoxynucleoside triphosphates, 0.5 µM of each of the virulence gene-specific primers, 1.5 U of Taq polymerase (Sigma) and 5 µg of template DNA. The amplifications were carried out in a Mastercycler gradient thermal cycler (Eppendorf) with 35 cycles of a denaturation step at 94°C for 60 s, primer annealing at 58°C for 60 s and extension at 72°C for 60 s. Furthermore, the extension time was done for an additional 3 s per cycle and a final extension step of 5 min at 72°C was performed. The negative control (no template DNA) was distilled water. The PCR products were detected by electrophoresis of 20 µL of each amplification mixture in 2% agarose gel in 1% Tris-acetate-EDTA buffer, after which the gel was stained with ethidium bromide (0.5 µg ml^{-1}).

RESULTS AND DISCUSSION

During this study, 100 samples of brain, kidney, spleen and liver were investigated. The results indicate that 20 of the 100 isolates are Gram positive cocci. Standard biochemical tests were also performed for identification of the isolates according to Austin and Austin (1999). Identification of the strains was done by multiplex PCR and 10 isolates were found to be *L. garvieae* and *S. iniae* (Figure 1). The results reveal that 28 samples were *S. iniae* and 12 samples were *L. garvieae*. After PCR, the 1100 and 870 bp bands were blasted with other sequences associated with *S. iniae* and *L. garvieae* in the gene bank (NCBI, Gen Bank).

Molecular diagnostic techniques such as PCR assays are increasingly used to detect and identify important bacterial fish pathogens including *Yersinia ruckeri*, *Pseudomonas anguilliseptica*, *L. garvieae*, *Renibacterium salmoninarum* and *Aeromonas salmonicida* (Miriam et al.,

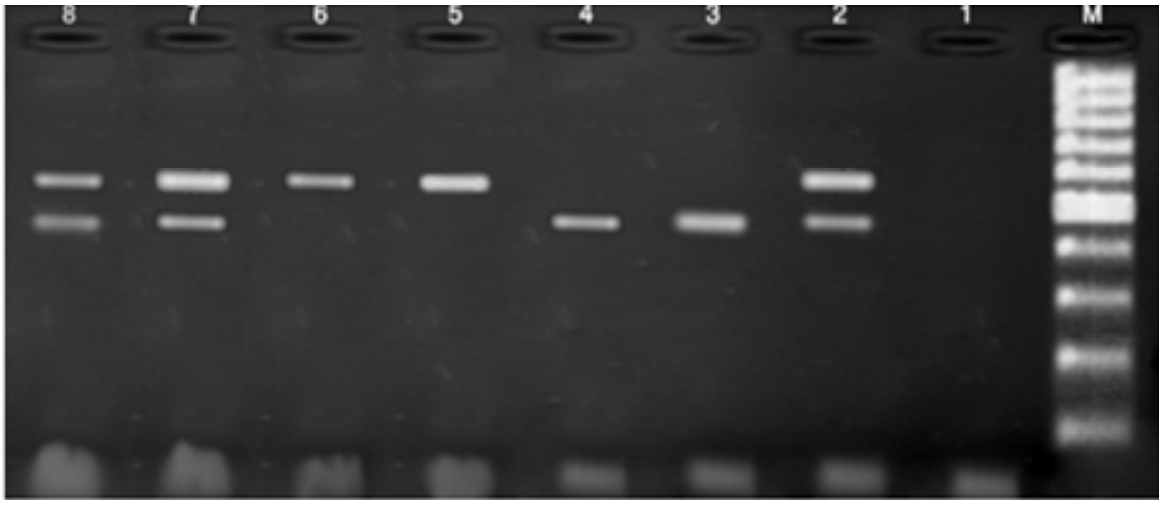

Figure 1. Amplification products from infected rainbow trout using multiplex PCR assay for the detection of *S. iniae* (870 bp) and *L. garvieae* (1,100 bp). Lane M, 100 bp DNA ladder; lane 1, negative control; lanes 2, 5 ,6, 7 and 8, *L .garvieae* positive in fish tissues; lanes 2, 3, 4, 7 and 8, *S. iniae* positive in fish tissues.

1997; Gibello et al., 1999; Aoki et al., 2000; Blanco et al., 2002). Mata et al. (2004b) used the lactate oxidase-encoding gene (lctO) from the *S. iniae* as a target molecule to identify and detect this pathogen by a PCR-based method. Also, Aoki et al. (2000) used a dihydropteroate synthase gene as a target for species-specific PCR of *L. garvieae*. Streptococcosis is one of the important infectious diseases that can lead to a lot of damages and mortalities in freshwater, brackish and salt water fishes. Zlotkin et al. (1998) used the species-specific PCR succeed to identify the beta hemolytic strains of *S. iniae* in sea bream and sea bass in the Mediterranean Sea. Moreover, Brunt and Austin (2005) showed that probiotics can be used to control the Lactococcosis and streptococcosis in rainbow trout. These immunostimulant materials lead to innate immunity and in particular the increase in the number of leukocytes and enhanced phagocytic and respiratory burst activity.

According to the aim of this study, we isolated the cocci from bacteria earlier approved through classical bacteriology and biochemistry tests and finally confirmed by using the multiplex PCR method. These bacteria both in the mediums and fish tissues were detected and identified. Simultaneous detection of the two mentioned bacteria in the rainbow trout farms indicated the involvement of important agent that lead to streptococcosis in fishes. This should be considered by fish health and epidemiological aspects. More also, Akhlaghi and Mahjor (2004) by evaluation of 225 samples from fishes suspected of having streptococcosis disease using bacteriological and biochemical tests, identified the *S. iniae* in the Fars province. In addition, Hussein and Hatai (2006) have emphasized on importance of *L. garvieae* as a serious pathogen in aquaculture industry and its impact on the production rate. They also have mentioned the involvement of human specimens that have contact with fish farms. Similarly, Mata et al. (2004a) used multiplex PCR in order to identify the four important causative pathogens of warm water streptococcosis: *S. iniae*, *S. difficilis*, *S. parauberis* and *L. garvieae*, so that four pairs of specific oligonucleotide primers were used for each of these pathogens. These researchers succeed in detecting pathogens in affected tissues and bacterial mediums. Shoemaker et al. (2008), while studying fishes infected by Gyrodactylus sp. suggested the occurrence of streptococcosis disease, which is caused by damage of the epithelial tissues thereby increasing the entering of bacteria into the fish's body.

Conclusion

Streptococcosis generally occurs in the warm seasons, especially in summer when the water temperature is high. Therefore, for rainbow trout cultured in cold water, prevalence of disease has a low rate unless the water temperature rises or environmental stressors which can cause the occurrence of the disease. In the present study, we succeeded in detecting and identifying two major species of streptococcosis disease by multiplex PCR technique in western part of Iran. Further attempts for molecular identification of these strains and introduction of new species are however needed.

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