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PCR Assay for Experimental Detection of *Streptococcus agalactiae* Based on *ScpB* Gene in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: *Streptococcus agalactiae* (Group B, GBS) is an important human and animal pathogen that causes invasive infection in fish. Present research was conducted by PCR experimental detection of *S. agalactiae* using *scpB* gene. One group of 48 fishes were challenged intramuscular (i.m.) with 0.5 mL bacterial suspension containing of 3.5×10^7 CFU/0.5 mL with normal saline and compared with control group that were injected by 0.5 mL sterile saline. Kidney tissue was selected for testing. Results showed that in 26 days, mortality ranged was 9.37% in challenged group. *S. agalactiae* was successfully detected by PCR in 42 challenged samples (87.5%).

Key words: *Streptococcus agalactiae*, *Oncorhynchus mykiss*, *scpB* gene, rainbow trout, rainbow trout, mortality, Iran

INTRODUCTION

Among the bacterial disease streptococci increased with the intensive farming of fresh water fishes. *Streptococcus* sp. is considered the main pathogen present in different fish species *Streptococcus agalactiae* is the most commonly found species in hot climate being associated to different fresh water, marine and estuary fish species (Evans *et al*., 2002). First reports of streptococci in fishes were from Hoshina *et al*. (1958) in Japan in cultivated rainbow trout. Science then, infection from this pathogen has been reported in several other fish species (Inglis *et al*., 1993).

The disease spread rapidly and caused severe economic losses in the farmed fish (Eldar *et al*., 1994). *S. difficile* was first reported by Eldar *et al*. (1994) as a non-hemolytic coccus causing septicemia and meningeocephalitis in rainbow trout. Recent studies have shown that *S. difficile* belongs to Lancefield's B group, Ib type with cellar protein electrophoretic profile indistinguishable from *S. agalactiae* (Vandamme *et al*., 1997). Kawamura *et al*. (2005) proposed a re-classification of *S. difficile* in order to unify the terminology, considering *S. difficile* and *S. agal* actiae synonyms. Eldar *et al*. (1995) reported the erratic swimming, decrease in appetite, lethargy, exophthalmia with intraocular hemorrhage and opaqueness of cornea and ascites in the fishes infected by *S. difficile*. Swimming in circles, uncoordinated movements and dorsal rigidness are indicators of the compromising of Central Nervous System (Duremdez *et al*., 2004). Disease management and assessment of cultured fish is a major concern to commercial aqua culturists.

The ability to identification of the presence of absence of a pathogenic organism in fish quickly would have significant economic benefits. An additional benefit would be if the concentration of the infectious organism could be determined in the fish or the environment so that changes in abundance of these organisms could be monitored. Detection and identification of the causative agent in fish samples based on bacterial isolation is difficult because of the lack of selective media and the fastidious nature (Bondad-Reantaso *et al*., 2005).

Finally, the development of a system that could accurately assess the carrier state of fish within an area containing a disease causative agent would aid in the development of management programs. Great advances have been made in improving the sensitivity and specificity of diagnosis of bacterial viral and parasitical fish. More commonly, DNA is amplified by the Polymerase Chain Reaction (PCR) using specific primers for diagnostic sequence (Prichard, 1997; McKeand, 1998). The PCR Technique as a molecular diagnosis tool has facilitated the detection and identification of an increasing number of bacteria of clinical significance in veterinary and human medicine (Altinok *et al*., 2001). It is the first time that *scpB* gene is used at fishes to diagnosis of this bacteria although, this method was used before in human (Dmitriev *et al*., 2004). The chromosome of Group B Streptococci (GBS) contains a gene which is related to the

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C5a peptidase gene (*scpB*). The *scpB* gene encodes a surface-associated peptidase which specifically C5a, a major chemo attractant generated in serum by activation of complement. The scpB encodes an open reading frame of 3,450 bp which correspond to a deduced protein (scpB) of 1,150 amino acids with a molecular weight of 126, 237 Da (Chmouryguina *et al*., 1996). The objective of the present study is to experimental detection of *S. agalactiae* in rainbow trout by using amplification of *scpB* gene.

MATERIALS AND METHODS

Fish: The 96 rainbow trout (*Oncorhynchus mykiss*) with an average weight of 900-950 g which were provided by a farm in the Haraz river were used in this experiment. These fish did not present any sign of infection neither by streptococci nor other pathogens. Before the beginning of each experiment, six fish were randomly used for PCR, cranial kidney and brain were collected for the PCR examination with the objective of verifying if the fishes were free from *S. agalactiae* and others.

Fish handling: The fishes are divided to 2 groups, each group 48 animals one of the groups was used as a control. These groups were kept in six 1000 L fiber glass containers with 900 L water and renewal rate of 9 L water min^{-1}. These containers were kept in the same conditions. The fishes were fed twice a day with extruded ration 3% live weight day^{-1}.

The fishes were acclimatized during 10 days before the beginning of the experiments. The containers were filled with continuous flow and aeration and cleaning was performed by suction on a daily basis. During the experiment average temperature and concentration of oxygen were 12±1°C, 8.7±0.5 mg L^{-1}, respectively. Oxygen levels were measured daily with equipment WTW (oxi3205, Germany).

Preparation of inoculums: *S. agalactiae* RTCC2051 strain was prepared from Department of Microbiology, Razi Vaccine Research and Serum Institute used as inoculums in the experiment. The isolated strain was aliquoted and stored in glycerin liquid on -80°C until used. A colony from blood agar pure culture of *S. agalactiae* separated and cultured in BHI (Brain Heart Infusion) and incubated in 37°C for 24 h.

To determination of CFU, 10-fold serial dilution of bacteria was cultured in blood agar culture and incubated in 37°C for 2 4 h then determined by colony counting. For the experimental infection, bacteria were routinely grown on BHI and were incubated in 37°C for 24 h washed in sterile saline then resuspended in sterile saline.

Experiment: The experiment was performed in a 26 days period. After 10 days adaptation, fishes were challenged with intramuscular injected (i.m.), midpoint between the dorsal fin and the lateral line with 0.5 mL bacterial suspension containing of 3.5×10^7 CFU/0.5 mL prepared with normal saline and the control group were injected by 0.5 mL sterile saline. After injection, the fishes were monitored once every 3 days for a period of 14 days. Collected or dead fishes were putted in sterile plastic bags for the macroscopic diagnosis, necropsy and bacteriological detection.

Collection and analyze of samples: Daily 6 fishes were captured randomly from each container for PCR test after 48 h of injection. A total of 96 samples after collection were autopsied immediately and kidney collected for PCR test.

Extraction of DNA from samples for PCR: The kidneys were removed from sampled rainbow trout and were homogenized then 150 μL PBS was added to samples (150 mg) of tissue homogenates. Samples were centrifuged at 3,000 RPM for 5 min and supernatant was transferred into a new micro-tube and centrifuged again at 13,000 RPM for 15 min. After that equal volume of PBS was added to the samples and 50 μL lysozyme added into the tubes then the tubes incubated in 37°C for 15 min. Bacterial cells were lysed with 4 h incubation at 56°C with lysis buffer (50 mM Tris-HCL, 1% SDS, 100 mM NaCl, 50 mM EDTA, 20 mg mL^{-1} proteinase K, pH 8).

The resultants lysates were extracted with saturated phenol, phenol-chloroform (50:50) and absolute chloroform, respectively. The tubes were centrifuged in 13,000 RPM for 15 min after each stage. The samples were purified with ice-cold ethanol (96°C) and incubated in -20°C for 20 min. After centrifugation 1,300 RPM in 15 min, 200 μL 0f 70% ethanol was added into the tubes then the tubes were centrifuged in 13,000 RPM for 5 min and liquids discard from tubes softly. Finally, the tubes were completely dried on a hot plate and 50 μL PCR grade water was added into the tubes then DNA stored at 4°C.

DNA primers for PCR-based assay for *S. agalactiae*: DNA primer pairs were selected from the published *scpB* gene encoding for C5a peptidase (Dmitriev *et al*., 2004). The nucleotide sequences of the primers were

forward (position 217-238) (P_2) (5′-ACAATGGAAG GCGCTACTGTTC-3′), reverse (position 471-450) (P_3) (5′-ACCTGGTGTTTGACCTGAACTA-3′). The primer pair P_2-P_3 were chosen to amplify a 254 bp segment (nucleotides 217-471 inclusive).

PCR assay for *S. agalactiae*: Amplification was performed in 25 μL containing PCR buffer, 2.5 mM; $MgCl_2$, 2 mM; dNTPs, 0.5 Mm; Taq polymerase (Cinna Gen Co Taq DNA polymerase 2,500 U (5U mL^{-1}), 1 U; forward primers 10 pm reverse primer, 10 pm and various concentration of DNA as the template (in a 10 μL volumes). The reaction was mixture by vortex. The PCR program were 94°C for 3 min followed by 30 cycles of 94°C for 1 min (denaturing), 48°C for 1 min (annealing) and 72°C for 1 min (extending) and a final incubation at 72°C for 10 min. The reaction was carried out in a eppendorf master cycler gradient.

These conditions were determined by varying the $MgCl_2$, nucleotide triphosphate and primer concentrations and the annealing and extension temperatures and times to maximize the amplification of the desired products and to minimize the appearance of secondary products. The amplified DNA products (10 μL volumes) were separated by electrophoresis in 1.8% low-melting temperature agarose gel, containing ethidium bromide at 100 V and the PCR product band was excised.

RESULTS AND DISCUSSION

Clinical signs was not seen in captured fishes until the 15th day whenever two fish was seen with dorsal rigidness at this day that these fishes was captured for considered. Five, three and one dead samples were recorded in 9, 10 and 11th days, respectively. These fishes were collected for considered. No dead sample was recorded in 12 days after this time sampling was done every 12 h until the end of period so that the 6 fishes at every turn were captured.

In the present study, kidney tissue from 96 rainbow trout fishes were tested for diagnosis of *S. agalactiae* by using PCR. DNA fragment of 254 bp were amplified from DNA extracted from rainbow trout kidney tissue of *S. agalactiae* by using primer pair P_2-P_3. These amplified products were consistently detected on agarose gels by using DNA extracted. At 4th day, a positive sample was recorded that clinical signs was not seen in this fish and then all samples are positive (Fig. 1). Out of 48 tested kidney samples of *S. agalactiae* were detected by PCR in 42 sample (87.5%) samples were PCR-positive and 6 samples were PCR-negative. The PCR-negative samples were captured in the early days after injection.

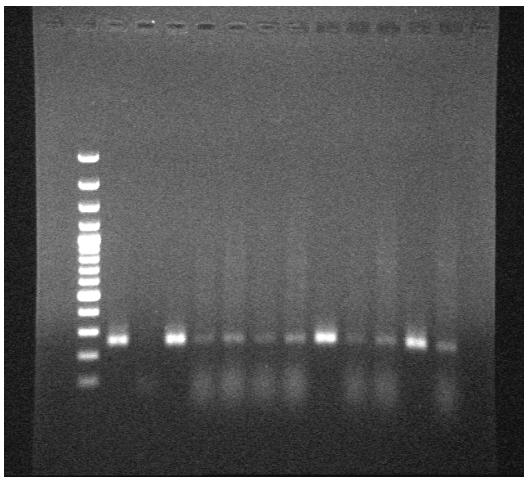

Fig. 1: Results of the *scpB* gene based PCR for used in the study. Lane 1, 100 bp ladder (fermentas); lane 2, positive control, (*S. agalactiae* RTCC 2051); lane 3; negative control; lanes 4-13, experimental samples

Total 46 samples of control group were all PCR-negative. Based on the results, the PCR assay for the *scpB* gene of *S. agalactiae* is a reliable, specific and sensitive method for accurate identification of this micro-organism isolated from different sources. In addition, PCR was effective in detecting *S. agalactiae* from inoculated tissue homogenates. The direct use of this PCR assay in tissue of diseased fish would allow the detection and identification of this bacterium within the same day of receiving a sample in comparison with the 2-3 days requirement for its isolation and further identification by conventional microbiological approaches.

Under ideal environmental conditions, healthy looking fish without a clinical sign or lesion can carry pathogens that create serious risks for the spread of contagions disease in the fish populations. Disease becomes evident only when stressful conditions the risk of stress increases and significant proportion of the stock may become infected. Therefore, detection of pathogen from carrier fish is essential for the effective fish disease control. The use of molecular diagnostic techniques such as PCR assays are increasingly to detect and identify many different bacterial pathogens including the most significant fish pathogens such as *Yersinia ruckeri*, *Pseudomonas anguilliseptica*, *L. garvieae*, *Renibacterium salmoninarum* or *Aeromonas salmonicida* (Blanco *et al*., 2002; Gustafson *et al*., 1992; Miriam *et al*., 1997; Gibello *et al*., 1999; Aoki *et al*., 2000). Although most of these PCR-based assays target specific sequences of the *16S rRNA* gene (Eldar *et al*., 1995; Berridge *et al*., 2001), other genes can also be used as

target molecules (Gustafson *et al*., 1992; Aoki *et al*., 2000; Pretto-Giordano *et al*., 2010). Many of the PCR assays use the *16S rRNA* gene as target molecule (Gibello *et al*., 1999; Blanco *et al*., 2002) sometimes among phylogenetically related microorganisms, it is difficult to design a specific set of primers for the identification of bacterial species based on the 16S rDNA.

Thus, the high genetic relatedness between *S. iniae* and *S. difficilis* (Vandamme *et al*., 1997; Berridge *et al*., 2001) may explain the non-specific amplification observed with the strains of the later species. This lack of specificity in the 16S rDNA PCR assay could be especially important taking into consideration that *S. difficilis* is also a significant fish pathogen that can be found concurrently with *S. iniae* (Berridge *et al*., 2001). The *scpB* gene should be an appropriate target molecule for the development of a specific *S. agalactiae* PCR assay.

Experimental challenge studies with *S. agalactiae* in fish have been usually focused on the determination of the DL50 of this pathogen and the mortality rates obtained after different inoculation doses and/or routes of administration (Pretto-Giordano *et al*., 2010). The detection limit in inoculated tissues homogenates of the PCR assay was lower than the tissue bacterial counts that *S. agalactiae* can reach in naturally diseased fish suffering from streptococosis which are in most cases, higher than 1×10^5 cells g^{-1}. Thus, it is likely that the PCR would be also useful for the rapid and precise diagnosis of natural infections by *S. agalactiae*. Evans *et al*. (2002) suggested that *S. agalactiae* isolates from fish are highly virulent and can infect a wide variety of freshwater and saltwater fish. Although, the incidence is not high, the severity of *S. agalactiae* infections makes necessary a rapid and accurate identification of this pathogen. Conventional biochemical methods may fail to identify *S. agalactiae* from clinical samples which can be misidentified with other biochemically related bacterial pathogens or causing similar clinical presentations. Thus, the PCR assay can be also a useful tool for rapid diagnosis of *S. agalactiae*.

CONCLUSION

Future analysis of suspected *S. agalactiae* organisms isolated from naturally diseased fish will allow determine the potential value of this scpB-based PCR assay for the accurate diagnosis of *S. agalactiae* infections.

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