MULTIPLEX PCR FOR DETECTION OF BACTERIAL PATHOGENS ASSOCIATED WITH STREPTOCOCCOSIS IN NILE TILAPIA

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Abstract

The intensification of tilapia culture system during last decade has led to disease emergence which is responsible for high economic losses in aquaculture. The most important pathogenic group in tilapia is *Streptococcus* spp., a causative agent for streptococcosis. This bacterial pathogen has several closely related species and can be able to infect wide ranges of host. Identification of streptococcal bacteria has previously relied on Lencefield serogrouping and biochemical test which are relatively time-consuming and difficult due to insufficient pathogenic database in currently available commercial test system. The molecular approach of polymerase chain reaction (PCR) can facilitate these problems. In this study, multiplex PCR assay was developed to identify 3 streptococcal species: *S. agalactiae, S. iniae* and *S. porcinus*, obtained from natural outbreak and experimental infection. First two species are known as main pathogens for tilapia streptococcosis, while the last selected bacteria is streptococcosis-associated pathogen in swine. This simultaneous detection of multiplex PCR could provide rapid, accurate and simple method for routine diagnosis and can be employed to evaluate the transmission of streptococcal species in different animal hosts.

Keyword: multiplex PCR, tilapia, *Streptococcus* spp.

Introductions

Bacterial infection is considered to be the most devastating disease as it can cause high mortality, thus consequently high economic losses are occurred. The possibility of outbreaks is increased if fish are stressed, as what happens with inappropriate water temperature, low dissolved oxygen, high nitrite levels, and high culture densities (Bunch and Bejerano, 1997; Perera et al., 1997; Shoemaker et al., 2000).

Streptococcosis has become a major problem for tilapia. Streptococcus is a gram-positive, non-acid fast, non-motile, oxidase-positive, catalase-negative cocci bacterium. These bacteria have ability to infect wide ranges of host such as human, swine, chicken and fish. The main pathogenic species responsible for streptococcal infections in fish are *Streptococcus agalactiae* and *S. iniae* (Eldar *et al*., 1994), while *S. porcinus* is streptococcosis associated pathogen in swine.

Clinical signs and gross morphological changes caused by *S. agalactiae* and *S. iniae* were similar. Typical signs observed included swimming in isolation prior to erratic swimming behavior, loss of buoyancy control, darkening, exophthalmia, corneal opacity, haemorrhages in or around the eye, the gill plate, base of the fins, anus, or elsewhere on the body. In some cases, the fish may show no obvious signs before death (Al-Harbi, 1994; Cook and Lofton, 1975; Eldar *et al*., 1994; Kitao *et al*., 1981; Miyazaki *et al*., 1984).

A diagnosis of bacterial infection in aquatic animal has been based on the microbiological analysis using bacteriological culture, morphological characteristic and biochemical tests. However, the identification of some Streptococcal species isolated from fish via a commercially biochemical test kit could not be possible due to lack of database. Therefore, the development of molecular technique such as Polymerase Chain Reaction (PCR) is become very useful for pathogenic bacterial detection and identification. An individual PCR assay using a single primer set has been widely applied for disease diagnosis. However, this technique can be relatively expensive and time-consuming when employed to detect large number of samples or several pathogenic diseases. Therefore, a multiplex PCR approach has been developed to overcome these weaknesses. The multiplex PCR can simultaneously detect several pathogens, hence cost-effective and relatively rapid results (Mata *et al*., 2004). Therefore, the objective of this work was to develop a multiplex PCR assay to detect a causative agent of streptococcosis disease which causes high mortality and economic losses in tilapia.

Materials and methods

Bacterial isolation and DNA extraction

Bacterial isolates of *Streptococcus* spp. from infected tilapia during the outbreak in 2007-2009 were collected and used in this study. *S. agalactiae* DMST 17129, *S. iniae* DMST 18775 and *S. porcinus* DMST 18780 were used as positive controls. All bacterial strains were grown on Tryptic Soya Agar (TSA) plates (Oxiod) for 24 h at 29°C. Purified bacteria were observed under microscope after Gram's staining. The biochemical properties of Genus *Streptococcus* sp. were identified and confirmed using API 20 STREP (Biomerieux, France).

Bacterial DNA was extracted following the method as previously described by Boom *et al*., (1990). Briefly, bacterial colony was added into 1.5 ml tube contained 500 μl phosphate buffer saline (PBS, pH 7.2) and 10 mg/ml proteinase K. After that, 1000 μl of L6 extraction buffer and 20 μl of Diatom were added into the tube, briefly vortexed and incubated at room temperature for 10 min. Tube was centrifuged at 13,000 rpm for 30 sec and supernatant was discarded. The pellet was subsequently washed twice with washing buffer L2, twice with 70% ethanol and 1000 μl of acetone once. After disposal of the acetone, the pellet was dried at 55°C for 10 min. TE buffer was added to dissolve the pellet, briefly vortexed, and incubated at 55°C for 10 min. After that, tube was briefly vortexed and centrifuged at 13,000 rpm for 2 min before downstream using or the DNA can be stored at -20°C until use.

Multiplex PCR

Multiplex PCR was performed in 25 μl reaction volumes using *Taq* polymerase (Invitrogen). The procedure is following manufacturer instructions, together with 0.4 μM of each primer (see detail in Table 1), 200 ng of DNA and water to bring the reaction volume to 25 μl. The multiplex PCR was performed on a thermocycler (Biometra) with the following thermo-profiles: an initial denaturation step of 94°C for 4 min; 35 serial cycles of a denaturation step of 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 s; and a final extension step of 72°C for 4 min. A negative control (water) and positive controls (DNA from *S. agalactiae* DMST 17129, *S. iniae* DMST 18775 and *S. porcinus* DMST 18780) were included in each batch of PCR. The amplified products were detected by electrophoresis on 1.5% agarose gel and visualized under UV light.

Table 1 Primer sequences for multiplex PCR

Species	Primer name	Sequences (5' to 3')
S .agalactiae	SagF	GGTGTTTACACTAGACTGATG
	IMOD	ACCAACATGTGTTAATTACTC
S. iniae	LOX-1	AAGGGGAAATCGCAAGTGCC
	LOX-2	ATATCTGATTGGGCCGTCTAA
S. porcinus	SpoF	GCACCAGTCTAATGAGTTGC
	SpoR	GTTACCGTCACGTAATGG

Challenge experiment

Native Nile tilapia (*Oreochromis niloticus*), weight between 40 – 60 g, were challenged in aquarium by injection. One hundred and tewnty tilapia were separated into two groups (treatment and control group, three replicates each). Parasite and bacteria contamination were examined prior experiment. *S. agalactiae*, *S. iniae* and *S. porcinus* were cultured in TSA at 29 ºC, 24 h for phenotypic and biochemical identification. Single colony each from three strains of *S. agalactiae*, *S.iniae* and *S.porcinus* were selected and cultured in Tryptic Soya Broth (TSB) at 29 °C, 24 h. The prepared broth cultures of each isolate were adjusted to an optical density (OD) of 0.1 at 560 nm using a spectophotometer to give final concentrations of 10^8 colony forming units (CFU)/ml. Bacteria were intraperitonially injected at 0.1 ml/100 g body weight for treatments, while 0.1 ml of 0.85% normal saline was injected for controls. Brain, liver, spleen, kidney and gill were collected at 48 h and 7 days post injection for further re-isolation, confirmation and PCR analysis.

Results

Bacterial identification

The characteristic examination of bacteria obtained from farm streptococcosis outbreak and experimentally infected fish was gram-positive, cocci, oxidase-negative, catalase-negative. Results from API 20 strep identification system showed negative for Pyrrodilonyl arylamidase, Inulin and Arabinose reactions, while positive results were detected from Acetoin production, β-Giucurodinase, Alkaline phosphatase and Ribose reactions. (Table 2). However, the identification of *S. iniae* using API 20 strep system can not be achieved due to lacking of database in this species.

Table 2 *S. agalactiae, S. iniae* and *S. porcinus* characterization using API 20 Strep

Characteristic	***S. agalactiae***	***S. iniae***	***S. porcinus***
Gram Strain	**positive**	**positive**	**positive**
Oxidase	-	-	-
Catalase	-	-	-
Acetoin production	+	+	+
Hydrolysis (hippuric acid)	+	-	-
β-Glucosidase hydrolysis (esculin)	-	+	-
Pyrrodilonyl arylamidase	-	-	-
α-Galactosidase	+	+	-
β-Giucurodinase	+	+	+
β-Galactosidase	+	+	-
Alkaline phosphatase	+	+	+
Leucine aminopeptidase	-	+	+
Arginine dihydrolase	+	-	-
Acidification (ribose)	+	+	+
Acidification (arabinose)	-	-	-
Acidification (mannitol)	-	+	+
Acidification (sorbitol)	-	-	+
Acidification (lactose)	-	-	+
Acidification (trehalose)	+	+	+
Acidification (inulin)	-	-	-
Acidification (raffinose)	-	-	-
Acidification (amidon)	-	+	-
Acidification (Glycogen)	-	+	-

Abbreviations: + = reaction occur , - = no reaction

Multiplex PCR from farm outbreak samples

DNA from 12 outbreak samples was examined using three pairs of primer set as above detail. Positive controls from *S. agalactiae, S.* and *S. iniae* produced bands at 122, 412 and 870 bp, respectively (Fig. 1) while no amplification was obtained from negative sample. Ten out of 12 tested samples was detected as *S. agalactiae* which consistence with the results obtained from API 20 Strep. *S. iniae* was found in one tested sample which previously could not be identified by using API 20 Strep. However, *S. porcinus* was not detected in this analysis which indicates that *S. porcinus* is not a causative agent for streptococcosis in fish from natural outbreak samples in our study.

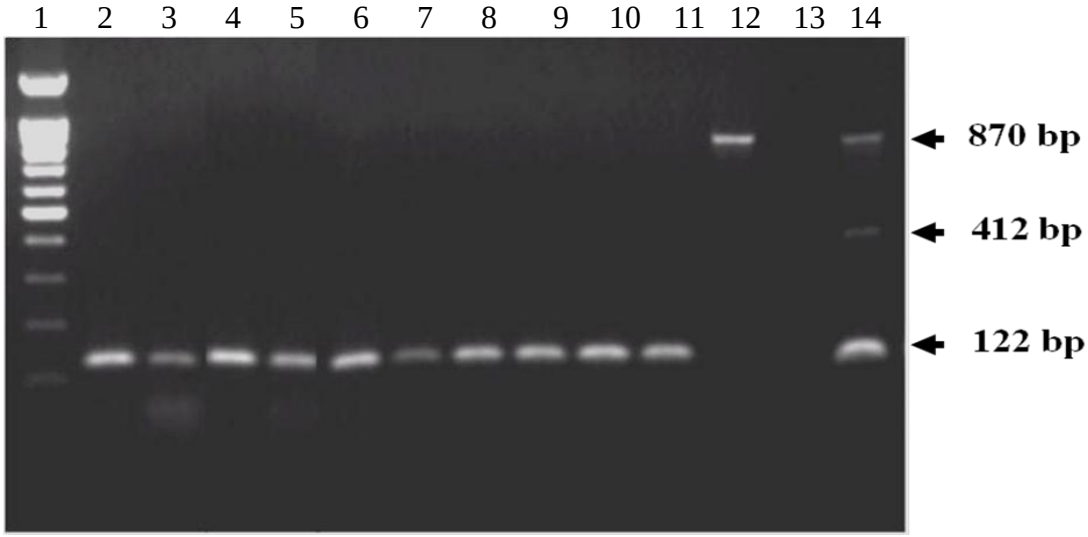

Fig1. The results of multiplex PCR analysis from fish samples in natural outbreak : Lane 1: 100-bp DNA ladder; Lane 2-11: *S. agalactiae*; Lane 12: *S. iniae*; Lane 13: negative control; Lane 14: positive controls of *S.agalactiae* DMST 17129 (122 bp), *S. porcinus* DMST 18780 (412 bp) and *S. iniae* DMST 18775 (870bp).

Multiplex PCR from challenge samples

The final concentration of three bacteria using in experimental infection of tilapia was 10^8 CFU/ml. Infected fish, collected after infection, were shown clinical signs compatible with streptococcosis such as erratic swimming, lethargy, darkening of the skin and enlargement of spleen. All bands from positive controls were also found as expected size: 122 bp for *S.agalactiae*, 412 bp for *S. porcinus* and 870 bp for *S.iniae* (Fig 2).

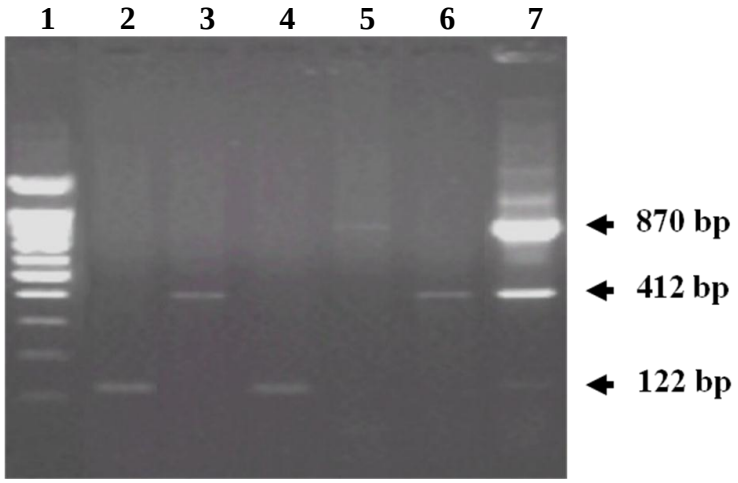

Fig2. Results of multiplex PCR from experimental infection, Lane 1: 100-bp DNA ladder; Lane 2 and 4: tissue sample from tilapia injected with *S. agalactiae*; Lane 3 and 6: tissue sample from tilapia injected with *S. porcinus*; Lane 5: tissue sample from tilapia injected with *S. iniae*; Lane 7: positive control *S. agalactiae* (122 bp), *S. porcinus* (412 bp) and *S. iniae* (870bp).

Discussion and conclusions

Streptococcosis is an important disease that has been reported in tilapia and cause high economic loss for the industry. The occurrence of streptococcosis in fish together with stress resulted in low grade chronic mortalities seen in some fish species, and acute mortalities of 30 to 50% occurring in others as a result of an encephalitis and systemic infection affecting multiple organs (Eldar *et al*., 1995, Camus *et al*., 2008). Streptococcus bacteria appear to have no specific host, and capable to infect both fish and mammalian species, for example *S. iniae*, *S. agalactiae* and *S. porcinus* being the most recently described member of this genus to infect an aquaculture fish species (Ringo and Gatesoupe, 1998; Shoemaker *et al*., 2007).

In general, the identification of bacterial pathogens for the routine diagnosis is the bacteriological culture and identification based on microbiological method of streptococcal infections in fish. However *S. iniae* could be misidentified in clinical microbiology laboratories with the remaining reported as "unidentified" by a commercial system. Up to the present, no commercial system has been found to include *S. iniae* in its database. Therefore, conventional biochemical methods may fail to identify *S. iniae* from clinical specimens. The use of molecular technique as a diagnostic tool for bacterial pathogens has become more frequently employed during the past few years. Thus, the development of multiplex PCR technique to detect bacterial pathogens associated with streptococcosis in Nile tilapia should be an effective tool for aquaculture industry, especially its capability to detect pathogenic specie that could not be able to identify from commercial system.

Multiplex PCR can be able to detect bacteria not only in pure culture but also from fish tissue. Moreover, the method would allow the detection of streptococcal pathogens without killing the aquatic animals by using gill tissue. This is an advantage over microbiological examination which needs to isolate bacteria only from internal organ of fish. Although, it is primer design is a drawback for multiplex PCR, but the assay provides rapid results, and cost-effective, comparing to microbiological method.

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