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Multiplex PCR assay for the simultaneous detection of bacterial pathogens in rainbow trout

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Abstract Accurate, rapid, and specific methods are required to identify pathogens for controlling bacterial diseases in fish. Various microbiological and biochemical methods are used to isolate and identify pathogens, but they are not adequately efficient and/or accurate. For example, distinguishing between *Streptococcus iniae* and *Lactococcus garvieae* is difficult via microbiological tests. Moreover, these bacteria often cause concurrent infections. Therefore, early and accurate identification of bacterial pathogens in trout is of utmost importance to prevent possible damage. Because of this, the multiplex polymerase chain reaction (multiplex PCR) method was optimized for simultaneous identification of bacterial infections caused by *Yersinia ruckeri*, *S. iniae*, and *L. garvieae*, three harmful bacteria of great importance in aquaculture. Multiplex PCR reaction was optimized on control samples, and the sensitivity and specificity of the test were evaluated. Multiplex PCR reaction was then performed on DNA isolated from samples collected from fish-breeding farms and transported to the laboratory. Multiplex PCR was found to be an accurate and sensitive method to identify simultaneously different species of bacteria.

Keywords Multiplex PCR · *Yersinia ruckeri* · *Streptococcus iniae* · *Lactococcus garvieae*

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Introduction

Rainbow trout is one of the most important commercial fish from the Salmonidae family, the distribution of which has a significant impact on the national economy. Today, infectious disease is one of the main limiting factors on the growth and development of the aquaculture industry. Bacterial diseases result in losses of more than $100 million worldwide each year (Sharifuzzaman et al. 2011).

Yersiniosis, streptococcosis, and lactococcosis are the most important aquatic diseases affecting farmed trout. Yersiniosis infection is caused by the *Yersinia ruckeri* in fish (Fernández et al. 2007). These bacteria cause septicemia, anorexia, numbness, ascites, splenomegaly, darkening of the skin, and bilateral exophthalmia (Altinok et al. 2001). More than one species of bacteria are involved in creating streptococcosis. Streptococcal infections also are a septicemic disease that causes high mortality (more than 75%) in the fish farming industry (Roach et al. 2006). Common causes of this disease are from the Streptococcaceae family and can cause disease in fish at any time during the year. Because this zoonotic disease has the potential for significant economic impact, it is therefore important to identify and treat these diseases (Mehrabi and RahimiKia 2013).

Isolation and identification of pathogens through rapid, sensitive, and specific methods are essential for controlling bacterial diseases. Various microbiological and biochemical methods are used to isolate and identify pathogens, but they are unable to distinguish similar bacteria. For example, distinguishing between *Streptococcus iniae* and *Lactococcus garvieae* is difficult through microbiological tests. Moreover, these bacteria often cause concurrent infections, making their identification costly and time-consuming.

However, the amplified DNA of microorganisms can now be used to detect the causative agent in suspect samples. Molecular methods such as PCR, especially based on 16S ribosomal RNA (rRNA) genes, are used for identification of gram-positive and gram-negative pathogenic bacteria (Altinok et al. 2008). Also, the lactate oxidase-encoding gene (lctO) is amplified as a target gene in PCR-based methods (Mata et al. 2004b). Molecular diagnostic techniques have higher sensitivity and specificity than do conventional laboratory methods.

Multiplex PCR is one of the best methods used for simultaneous identification of pathogenic bacteria in fish (Mata et al. 2004a; Del Cerro et al. 2002; Tapia-Cammas et al. 2011; Altinok 2011). The aim of this study was to optimize multiplex PCR reactions for the simultaneous detection of *Y. ruckeri*, *S. iniae*, and *L. garvieae* as important bacterial etiology in trout fish farms. For this purpose, PCR single reactions were optimized for each of the bacteria, and multiplex PCR test was optimized, for the first time, to simultaneously identify positive and negative bacteria in trout.

Materials and methods

The fish samples

Samples were randomly obtained from 16 cold-water rainbow trout farms in the Iranian cities of Faruj, Shirvan, Bojnord, Esfarayen, and Ashkhaneh, all of which are in the North Khorasan province (Supplementary Table 1). These farms were chosen because of disease records in a veterinary GIS system. The samples were collected from fish with or without clinical signs. The fish samples were transported to the laboratory in ice boxes and in less than 24 h time.

Bacterial cultures

First, for bacteriological culture, fish samples were aseptically split, using surgical scissors, from the rectal area to the anterior part, and then specimens were taken by sterile Pasteur pipette from the kidney, heart, spleen, and liver. The specimens were then cultured in Trypticase soy broth (TSB) medium and incubated at 30 °C for 24 h. In the case of opacity, streaking the plates from this medium was done on blood agar medium with 5% defibrinated sheep blood to obtain a single colony and, subsequently, incubated at 30 °C for 24 to 48 h. After the incubation period, macroscopic and microscopic examinations of colonies were performed by gram staining and culture on MacConkey agar medium.

Positive and negative control bacteria

Y. ruckeri, *S. iniae*, and *L. garvieae* were used for positive control. Bacteria samples similar to the positive control, both taxonomically and ecologically, were tested as negative controls (Supplementary Table 2).

DNA extraction from bacteria and tissues

The boiling method was used for extraction. The number of pure bacterial colonies was delivered to 100 μl deionized distilled water, then 900 μl of buffer A (Tris 10 mM, $mgcl_2$ 10 mM, sucrose 32%, Triton X-100 1% pH 7/5) was added, and after vortexing, it was centrifuged for 2 min at 10,000 rpm. This stage was continued until the emergence of white precipitate. The supernatant was discarded, and 100 μl of NaOH was added and boiled at 100 °C for 20 min. A cooling tube was used to add 20 μl of Tris buffer, and the solution was inverted a few times, and then it was centrifuged at 10,000 rpm for 2 min. The supernatant was transferred to a new tube, and cold ethanol at double fluid volume was added to it and kept at −20 °C for 20 min. Finally, 40 μl of Tris buffer was added to the extracted DNA and stored at −20 °C. DNA was extracted from the spleen, liver, and kidney tissues according to the Qiagen 69504 kit protocol. Agarose gel was used to assess the quality of the extracted DNA.

PCR conditions

PCR was performed in 50 μl volumes, incorporating 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 1.25 U of Taq enzyme (Takara, Clontech), and 5 μl of 10× reaction buffer. Reaction conditions consisted of 35 cycles of denaturation at 94 °C for 30 s; annealing at 55/50/60 °C for 40 s for *S. iniae*, *L. garvieae*, and *Y. ruckeri*, respectively; and extension was performed at 72 °C for 50 s. The primers, as described in the literature, are shown in Table 1.

Table 1 Primers used in PCR reactions

Primers	Reference
Yersinia ruckeri	Gibello et al. (1999)
Streptococcus iniae	Mata et al. (2004a, b)
Lactococcus garvieae	Zlotkin et al. (1998)

Multiplex PCR reaction

A range of concentrations of PCR components were tried in order to optimize PCRs. Primer concentrations of 0.05 μM to 1 μM, magnesium chloride concentrations of 0.5 mM to 5 mM, dNTP concentrations of 0.1 to 0.2 mM, Taq enzyme concentrations of 1 to 1.5 units, and DNA concentrations of 10 to 500 ng from any sample were used in a final volume of 50 μl. The number of cycles was 35, and a gradient temperature of 50 to 60 °C was used to obtain the annealing temperature.

Multiplex PCR specificity and sensitivity

Genomic DNA was extracted from positive control strains to examine the sensitivity. DNA concentration is estimated by measuring the absorbance at 260 nm with NanoDrop, and the DNA concentration of each bacterium was reached to 100 ng/μl. Then, accordingly, serial dilutions of 50 ng to 5 pg were prepared for each DNA sample. One microliter of any dilution was used for PCR. Bacterial DNA from the negative control was extracted to determine specificity, and after mixing, multiplex PCR reactions were performed on bacterial DNA.

Sequencing method

To confirm the PCR products, we purified products of multiplex PCR (mPCR) on farm samples. Both strands of the PCR products were sequenced. The results of the sequencing were analyzed and blasted for homology searches.

Results

Bacteriological tests

According to the study results, all gram-positive cocci were small catalase-negative white and mucoid colonies; beta hemolysis was very clear on blood agar. Catalase-positive and gram-negative bacilli also had small white and gray colonies (Supplementary Table 3).

Single PCR reactions

Molecular experiments were performed using primers listed in Table 1, based on the gene 16S rRNA for *Y. ruckeri* and *L. garvieae* and the gene lctO for *S. iniae*. PCR tests for each of the bacteria were optimized based on temperature and concentration of the components in order to observe single-band products on the gel with the desired size. Sizes of product were 590, 870, and 1100 bp for *Y. ruckeri*, *S. iniae*, and *L. garvieae*, respectively (Fig. 1).

Multiplex PCR reactions

The best results were obtained through a primer concentration of 0.4 μM for streptococcus and lactococcus, and 0.1 μM for Yersinia. Also, 2.5 mM magnesium chloride, 0.15 mM dNTP, and 1.25 units Taq enzyme for 50 μl were used. The concentration of DNA was 100 ng for streptococcus and lactococcus bacteria and 50 ng for yersinia that had been isolated from tissue

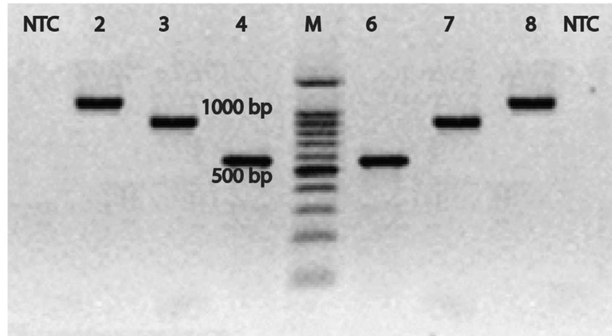

Fig. 1 The results of single PCR reactions on the control and test samples: *lanes 2* to *4* respectively belong to the positive control bacteria of *Lactococcus garvieae* (1100 bp), *Streptococcus iniae* (870 bp) and *Yersinia ruckeri* (590 bp). *Lanes 6* to *8* are also related to sample farms with codes of 1 and 3 and 13

samples. The number of cycles was 35, and the optimal annealing temperature was 55 °C for all three bacteria (Fig. 2). DNA test sensitivity was 5 pg from positive culture bacteria (Supplementary Fig. 1). The specificity of the assay was validated by setting the mPCR with DNA from taxonomically related bacteria with a high DNA concentration, and no band was observed (Supplementary Fig. 1, lane C). mPCR was carried out on samples from the farms, and most of them showed contamination with bacteria (Fig. 3 and Table 2). According to the sequencing results, the mPCR products were confirmed to belong to the target bacteria.

Discussion

In this study, multiplex PCR reaction was optimized for the identification of gram-positive and gram-negative bacterial DNA. With multiplex PCR, time-consuming and sometimes inaccurate microbiological and biochemical methods are no longer needed.

Yersiniosis, found worldwide, is considered an endemic disease in most countries producing farmed rainbow trout. Expenses related to losses, decreased growth, reduced feed-conversion ratio, antibiotics, and delayed harvest due to diseases make up about 20% of total production costs. Given the value of the aquaculture industry, definitive diagnosis of yersiniosis is of special importance in trout farms (Austin and Austin 2007). Streptococcosis is the leading cause of heavy losses, morbidity, and mortality in rainbow trout breeding. According to the results of this study, *L. garvieae* is the main agent causing streptococcosis in trout breeding. Importantly, because they are zoonotic agents, these bacteria may put human health at risk.

The multiplex PCR method was first used in 2002 to identify yersinia, aeromonas, and flavobacterium (Del Cerro et al.). Therefore, similar studies were carried out, and the multiplex

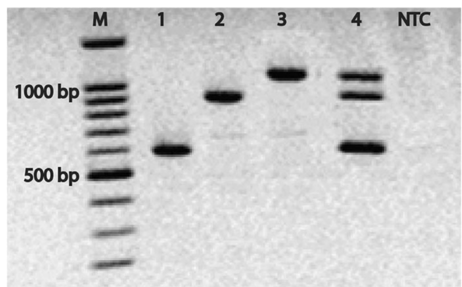

Fig. 2 Multiplex PCR reactions on positive control samples: *lanes 1*, *2*, and *3* single PCR and *lane 4* multiplex PCR on the same samples

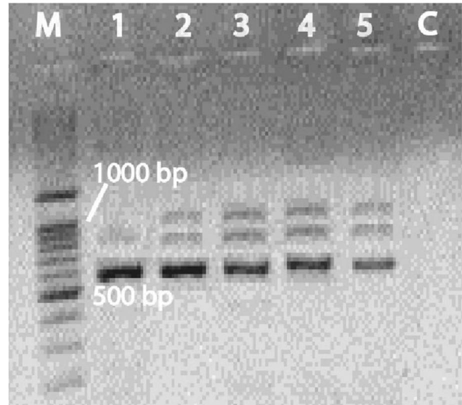

Fig. 3 The results of multiplex PCR reactions on samples from farms; *lane 1*: positive controls of three bacteria including *Yersinia ruckeri* (590 bp), *Streptococcus iniae* (870 bp), and *Lactococcus garvieae* (1100 bp); *lanes 2*, *3*, and *4*, respectively, belong to farms *1*, *2*, and *4* infected by *Y. ruckeri*. *Lanes 5*, *6*, and *7*, respectively, from farms of *5*, *8*, and *16* are related to *S. iniae* and *L. garvieae*; *lanes 8* and *9* from farms *11* and *13* infected by *L. garvieae*

PCR method was examined to identify bacteria in fish (Onuk et al. 2010; Zhang et al. 2014; Tsai et al. 2012). However, this is the first report of simultaneous identification of gram-positive and gram-negative bacteria, including *Y. ruckeri*, *S. iniae*, and *L. garvieae* using mPCR. These three bacteria are the main causes of infectious diseases in some regions that cause high mortality in farmed fishes, and identifying them is of great importance both from a sanitation and from an economic standpoint.

Identifying asymptomatic infected fish can prevent transmission of infectious agents to other fish and decrease the possible likelihood of damages. In this study, *Y. ruckeri* bacteria were identified in infected fish without clinical signs, as they were in Altinok et al.'s 2001) study.

Diagnosis of infection in farms by mPCR helps us to use appropriate antimicrobial agents to treat and control the disease. This technique is helpful in epidemiological studies for determinant focality of diseases and prevention of outbreaks. With timely and rapid diagnosis, losses and expenses can be diminished within the aquaculture industry.

According to the results, multiplex PCR is a useful, inexpensive, accurate, and rapid method for identifying bacterial agents in farmed trout in comparison to bacteriological testing methods. Therefore, standard microbiological methods can be replaced with multiplex PCR. mPCR is more sensitive than bacteriological cultures, and more samples can be identified by this assay in a shorter period of time.

Table 2 Identification of bacterial contamination of the farm samples through molecular methods

Clinical examination		Single PCR		Multiplex PCR	
	Number of farms	Pathogens	Number of farms	Pathogens	Number of farms
With signs	5	*Y. ruckeri*	5	*Y. ruckeri*	5
	6	*S. iniae*	–	*S. iniae*	–
		L. garvieae	3	*L. garvieae*	3
		S. iniae + *L. garvieae*	–	*S. iniae* + *L. garvieae*	3
Without signs	2	*Y. ruckeri*	2	*Y. ruckeri*	2
	3	No pathogen	–	No pathogen	–
Total	16		10		13

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