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Original Article

Development of 16S rRNA Targeted PCR Method for the Detection of *Escherichia coli* in Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Background & Objective: **The presence of *E. coli* in fish intended for human consumption may constitute a potential danger, not only on causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to those infecting humans. The objective of this study was to develop an improved PCR method based on species – specific 16 S rRNA gene primers (FES, RES) for detection of *E. coli* from agar plates and fish tissues.**

Materials and Methods: **For the rapid detection of *E. coli* from fish, a set of primers (FES, RES), targeting 16S rRNA gene sequences of the specific microorganism was designed, and fifty two rainbow trout were obtained. Then 1mL of bacterial concentration of 10^6 CFU/ml was injected into intraperitoneal cavity. Samples were collected from liver and kidney after 48h injection. The PCR reaction conditions were optimized to permit detection of organism from agar plates and fish tissue in a day.**

Results: **All tissue samples were positive for microbiological and PCR identification. DNA was successfully extracted by a boiled – extraction method or by phenol – chloroform – isoamyl alcohol. The BLAST analysis from sequencing of 4 amplicons randomly selected showed similar results, with the match being *E .coli* with a 100% similarity.**

Conclusion: **This method is fast, specific and sensitive to detect *E. coli* in infected and asymptomatic animals, fish product, and may have a positive impact on public and environmental health.**

Keywords: **16S Ribosomal RNA, *E. coli*, PCR, Rainbow Trout**

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Introduction

The fish is often exposed to various microorganisms. A number of these microorganisms naturally present in the environment, and some of them enter water via animal excreta, agricultural runoff and human wastes (1-3). Although *Escherichia coli* is not a normal inhabitant of fish flora, it has been isolated from the stomach and intestines of fish (4-7). This fact indicates that bacterial flora of fish would reveal the bacteriological conditions of the water where fish inhabit (8-10). *Escherichia coli* is one of the most important microbial pathogen in field of food safety and quality, with pathogenic potential for human and other animals. It causes mass mortalities in number during the previous years, which needs public health attention (11-16).

The presence of *E. coli* in fish for human consumption may constitute a potential danger causing disease, in addition can be cause of the possible transfer of antibiotic resistance from pathogenic fish bacteria to these infection humans (17-18). In aquaculture, increased number of antibiotic – resistant bacteria have been a consequence of the extensive use of antibacterial chemotherapy in the control of microbial diseases many of these carry transferable R – plasmids and thus the potential for further spread of resistance (19-21).

Transfer between pathogenic fish bacteria and *E. coli* has been intensively studied (22). Over the last several decades, the focus of research has expanded with the realization organisms are widespread in the environment. The imprudent use of agricultural and other food production associated applications of antibiotics selects for resistant organisms (23-26).

The spread of antibiotic resistant microorganisms in the environment is recognized as an important public health issue with physicians concerned their future ability to treat infectious diseases (27). Therefore, rapid and accurate identification of microorganisms have a significant impact on strategies and fish health management programs designed and improve the safety of the products(1).

In both developing and developed countries there have been sanitary control programs for monitoring and detection of fecal coliforms in farm, environment, processed fish or other fishery products (28-29).

Conventional diagnostic methods involve culturing bacteria on agar plates followed by phenotypic and immunological methods or histological examination (30). However, it might be difficult to detect some of the bacteria due to fastidious growth, morphological variations and unusual biochemical reactions. Molecular diagnosis methods such as reverse transcription PCR, quantitative PCR, real-time PCR, AFLP, RFLP and RAPD have been designed to detect specific nucleic acids without depending on the capability to culture or need to isolate the pathogen (31).

The 16S rRNA gene has highly conserved regions in all prokaryotic organisms and variable regions that have been used to provide valuable taxonomic information at various levels of classification and to distinguish between species and isolates (13, 32).

We aimed to develop an improved PCR method based on species – specific 16 S rRNA gene primers (FES, RES) for detection of *E. coli* from agar plates and fish tissues.

Materials and Methods

Samples

Fifty two rainbow trout with a mean weight of 246 g (±20/91) and mean length of 27/04 cm (±0/90) Were obtained from Karaj Fish Farm and housed at the fishery and environmental Department of Tehran University Aquatic Animals Laboratory.

The fish were stocked at a rate of 13 fish per l000/ l tank in 4 tanks. These fish were held for 2 weeks prior to experimentation in order to acclimatize them to tank conditions. Aeration was supplied to each tank with compressed air through air stones. Water was supplied at 0.5 l/min with daily dissolved oxygen of 7 ±0.7 mg/l and temperature of 14.5°C (± 1.5).

Bacteria

Bacterial isolates of strain number of RTICC 2325 used in the experiment were obtained from the Razi Vaccine & Serum Research Institute, Karaj, Iran.

For the challenges, the bacteria were grown on TSA (Himedia – M 290). At 37°C for 24 h and harvested in sterile physiological saline to 10^{-9} and diluted to on optical density of 1.0 at a wavelength of 640 nm. This corresponds to a bacterial concentration of 1 x 10^6 CFU/ml.

Challenge with E. coli

Before bacterial challenge 6 of the fish were randomly selected from two tanks and were anaesthetized in 100 mg/ml of tricaine methane sulphonate and were injected intraperitoneally with 1 ml of the bacterial suspension. Two 1000/l tanks containing 13 fish each was held as normal controls and were maintained as previously described. Fish were then returned to each treatment tank and allowed to recover from the anesthetic after challenge.

Fish sampling

After 48 h injection three fish from each tank of 13 fish were caught and killed. The body surface of the fish were swabbed using 70% ethyl alcohol to prevent contamination of the culture by normal external bacterial flora. From each fish, samples were collected from the kidney and liver. Liver and kidney were aseptically streaked on tryptic soy agar. Following incubation, 1 typical colony was selected from each isolates and sub cultured on to MacConkey and EMB to check purity of the isolate. All isolates were stored in a broth culture supplemented with 15% glycerol at -70°C. In order to obtain similarly sized samples, 1 mm cubes of liver and kidney were aseptically removed and put in microcenterfuge tubes for detection of micro organism from fish tissue.

Phenotypic characterization

Isolates were classified as *E. coli* according to their reactions in the following conventional tests: Catalase, motility, indole , voges – proskaues , urea , triple sugar iron (TSI) , glucose , methyl red , H_2S production , citrate utilization. Each substrate was incubated at 37°C and reactions read after 24 and 48 h.

DNA extraction

Template DNA was obtained from colonies on agar plates, and artificially infected fish tissue by a boiled – extraction method and by phenol – chloroform –isoamyl alcohol. For PCR a boiled method was used as follows: each isolate was grown at 37°C for 24 h on TSA. One colony was resuspended in 10 ml distilled water and was heated at 96°C for 10 min, and finally, rapidly cooled on ice. DNA concentrations of all samples were evaluated using a spectrophotometer. Template DNA for PCR was extracted form the following tissues: liver and kidney were mixed with an equal volume of lysis buffer (250 mM Nacl, 100 mM Tris – Hcl at ph 8.0 , 1% sodium dodecyl sulfate) and 30 ml of proteinase – k solution were incubated at 60 c for 3 h in a water bath.

After extraction of the sample with a mixture of phenol – chloroform – isoamyl alcohol (50:50),

DNA was precipitated with cold ethanol, pelleted by centrifugation, washed once with ethanol, and dried. Before using in the PCR, DNA was dissolved in the buffer (Altinok, 2008). DNA quality was evaluated using electrophoreses on a 0.8% agarose gel.

PCR primer design

The sequence of the 16S rRNA gene of *E. coli* was retrieved from the Gen Bank database (National Center for Biotechnology Information) and compared with the closest relatives employing the Mega BLAST soft ware (Table 1).

Table 1: Species specific PCR primer for *E. coli*

Primer	3′5-′(Sequence)
FES	**F:GGAAGAAGCTTGCTTCTTTGCTG**
RES	**R:AGCCCGGGGATTTCACATCTGA**

PCR condition

The PCR protocol was optimized by amplification reaction in a thermal cycler (Astec, Japan). Reaction mixtures had 1 of each primer, 1μL of the DNA template, 17.5μL of sterile distilled water, 1μL of Mgcl2, 0/5 of dNTP, and 2.5μL of PCR buffer. PCR conditions consisted of an initial denaturation step at 94 °C for 7 min followed by 30 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min).And a final 10 min elongation period at 72°C. Controls received the PCR mixture containing 1) No DNA template, 2) DNA from negative control fish, or 3) DNA from *E. coli* (Positive control).

Confirmation of the primer specificity

To verify that the specific primer-pair amplified *E. coli* DNA, the PCR product was purified with a PCR purification Kit (Qiagen) and directly sequenced with an ABI 3130 genetic analyzer (Applied Biosystems Instrument) in Avicenna Research Institute. The results of the sequencing were used for homology searches by the BLAST program (http://www.ncbi.nlm.nih.gov).

Results

Two species – specific 16 S r RNA gene primers (FES, RES) were designed (Table 1). The primers set amplified a specific 544 bp DNA fragment from *E. coli* strain (Fig. 1). Detection of the bacterium within DNA templates derived from liver and kidney was possible as early as 48 h after challenge in healthy carriers.

E. coli was detected from cultures on agar plates and fish tissues. Representative examples of product formation from each source are shown in Fig. 1. All tissue samples were positive for microbiological and PCR identification (Table 2). DNA was successfully extracted by a boiled extraction method or by phenol – chloroform – isoamyl alcohol. The total procedure was accomplished in a day. From the point of DNA extraction to observation in an agarose gel, and no amplification products were obtained from negative controls. To confirm that the positive PCR results occurred, we sequenced the amplified DNA products from different PCR reactions and DNA extractions. The BLAST analysis from sequencing of 4 amplicons randomly selected showed similar results, with the match being *E. coli* with a 100% similarity (data not shown). These finding suggest the high specificity of the primers to detect *E. coli*.

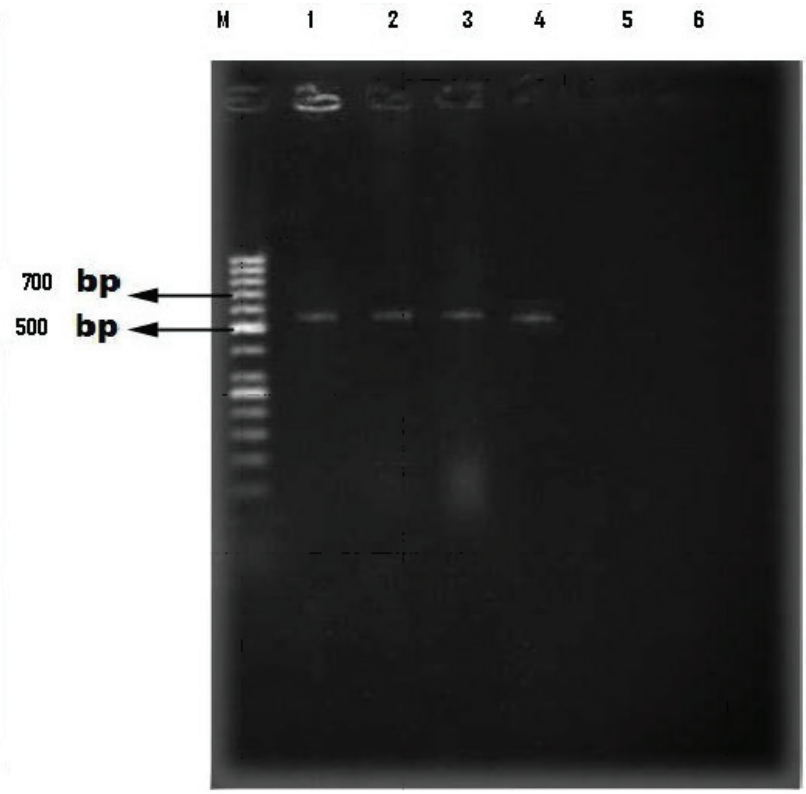

Fig. 1- Specificity PCR products from fish tissues, agar plate and positive control using FES-RES primer set for detection of *E. coli*. Lane M, molecular size marker; lane 1, E. coli RTICC 2325(544 bp); Lane 2, kidney; Lane 3, liver; Lane 4, bacteria isolated from agar plate; Lane 5 and6, negative control containing PCR from un injected fish tissue.

Table 2: Identification and characterization of *E. coli*

Characteristic of isolates identified as *E. coli* by both PCR and biochemical tests	Biochemical tests								Literature (Forbes ***et al.***1998)								Molecular diagnosis
	TSI	Cit	SIM	Nit	MR-VP	Cat	U	Gas/ glucose	TSI	Cit	SIM	Nit	MR-VP	Cat	U	Gas/ glucose	
KE-1	A/A	-	-++	+	+ -	+	-	-	A/A	-	-++	+	+ -	+	-	- 10٪ +	544 bp
LE-1	A/ K	-	-++	+	+ -	+	-	-	A/K								544 bp

Urea =U Catalase=Cat Citrate =Cit Gas from glucose =gas/g

Nitrate= Nit Sulfid,Indole,Motility=SIM MR-VP=Methyl red, Voges-proskau

TSI=Triple Suger Iron

Discussion

The fish is often exposed to various microorganisms. The numbers of these microorganisms naturally present in the environment, and some of them enter water via animal excreta, agricultural runoff and human wastes. Significant numbers of these such as *E .coli* remain on the skin and in the guts of fish and can pose a potential public health risk (33). Because of their great capacity to move, fish are able to carry potentially pathogenic bacteria for humans to non-polluted waters causing infection when fish are consumed or handled (24, 34).

But there have been relatively few studies about the presence of fecal coli forms and *E. coli* in fish. Studies of fecal coli forms in fish have been concerned mostly with their role in the spoilage process and contamination as the product. This study demonstrated a PCR specific technique that uses the 16 S r RNA gene to detect *E. coli* from liver and kidney as early as 48 h after challenge in fish. We found that the PCR primer set, FES-RES, was specific for the identification of *E .coli*, and differentiated *E. coli* from other fecal coli form. The DNA from other closely related bacterial species, as well as from a variety of other common pathogens, did not amplify in this PCR, which was supported by the sequencing results obtained. All tissue samples were positive for microbiological and PCR identification.

The detection of the pathogen after challenge in the skin, mucus, intestine and gill in this study is not surprising, because it can be hypothesized that some bacteria should superficially attach to and embed in these organs simply as a result of bathing the fish in a bacterial suspension. Liver and kidney samples were the most reliable tissue type for the detection of *E .coli,* because there are not any bacterial floras in these organs. We also found that this PCR primer set allowed detection *E. coli* from agar plates. This method is capable to detect culturable and also non- culturable but viable cells, which increases its sensitivity as a detection method. Furthermore, our data suggest that the combination of this sampling method and the FES-RES primer set was able to discriminate between apparently healthy and asymptomatic fish infected with *E. coli* and uninfected.

The fact that FES-RES primer set can detect symptomatic animals is not surprising, since animals with clinical signs of *E .coli* typically have enough bacteria to make a determinati*on* of etiological agent via culture. What is useful is the fact that using FES-RES primer set, *E. coli* can be detected in asymptomatic fish. Therefore, the FES-RES set may allow for earlier detection of *E. coli* before human consumption and may help in prevent spread of disease. To avoid contamination each of the following steps in the method was performed in a separate room: necropsy, DNA extraction, PCR master mix preparation, DNA quantification and addition to the PCR mixture, and PCR reaction and electrophoresis. Laboratory coats, gloves, pipettes, and other supplies were not moved between these rooms. New disposable razor blades, forceps, and gloves were used for each fish to reduce potential contamination between fish.

The PCR has been used successfully to detect and identify fish – pathogenic bacteria / including *Aeromonas hydrophila / a. salmoncida / Edward siella tarda /Renibacterium salmoninarum / Vibrio anguillarum* , v . *unlnificu*s and *yersinia rukeri* (3-7,9,11,20,22,24).

The primers set amplified a specific 544 bp DNA fragment from *E. coli* strain. The result is similar to those previously reported in the literature (31). This result supports the assumption penetration and establishment of bacteria in different tissues and organs of fish, such as digestive tract, gills, muscle, kidney, liver and gas bladder have been reported in polluted aquatic environments. Bacteria may break the immunological barriers of fish, penetrating different tissues and organs (30). And they may survive and accumulate in fish (11, 17, 35-37).

It has been showed that fecal coli forms and *Streptococcus facials* could be found in fish

intestines (20). They speculated that fecal coli forms are not part of the permanent micro flora in fish, but their presence is caused by polluted water and is in flounced by feeding habits. Guzman *et al.* (22) found two fish species that harbored *E. coli* from a river contaminated by sewage effluent. Their work also supported the view that fish obtund *E. coli* from the environment. French *et al.* (19) reported the isolation of high numbers of multiple – antibiotic – resistant *E. coli* from focally contaminated streams in Hong Kong. Mukherjee and chakraborty (27) found that the majority of resistant bacteria isolated from river water carried R – plasmid, which was transferable to other organisms. High levels of microbiological contamination may pose a potential public health risk if fish are harvested in the consumption of contaminated fish need not necessarily be associated only with the bacteria present in the edible tissues (17). Infection may also occur during handling of the fish, and cross contamination to other food sources is likely to occur when the fish is prepared and cleaned for consumption (34).

In addition, recent researches showed viable count of antibiotic resistant bacteria isolated from gill and intestinal content samples (including *E. coli*), may pose a risk to public health among fish consumers (26).

Conclusion

Designed 16S rRNA gene – based FES –RES primer set is a valuable tool for the detection of *E. coli.* It provides a rapid, reliable, specific and sensitive for detection that can be used directly by diagnosticians and fish farmers alike. These results, together with those obtained in fish tissues and agar plates indicate that PCR is capable of detecting culturable and also non- culturable but viable cells, which increases its sensitivity as a detection method. Therefore this type of *E. coli* detection in viable tissues can serve as a model diagnosis in other animals including human. The FES –RES set to detect *E. coli* in asymptomatic animals and fish production and may have a positive impact on public and environmental health.

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