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Original Article

Simultaneous detection of *Aeromonas hydrophila*, and *Escherichia coli* in Rainbow trout (*Oncorhynchus mykiss*) by Duplex PCR

Fatemeh Fattahi[1], Alireza Mirvaghefi*[1], Hamid Farahmand[1], Gholamreza Rafiee[1], Alireza Abdollahi[2]

[1]Department of Fisheries, Faculty of Natural Resource, University of Tehran, Karaj, Iran.
[2]Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Abstract: Rapid and accurate identification of microorganisms have a significant impact on strategies and fish health management programs. Hence, in this study a duplex PCR assay based on the 16s rRNA gene for simultaneous detection of *Aeromonas hydrophila* RTICC 1032 and *Escherichia coli* RTICC 2325 from pure cultures, and challenged fish tissues was performed and their results compared with the results of single PCR assays for each bacterium. For this purpose, an experiment with three treatments including artificially infected with *A. hydrophila*, *E. coli* and a mixture of them with a control group was designed. Fish were injected intraperitoneally with 1 ml of sterile physiological saline containing 10^6 CFU/ml of the corresponding bacteria. Samples were collected from liver, kidney and spleen 48 hrs post-injection. A duplex PCR based 16S rRNA genes was developed for the simultaneous detection of *A. hydrophila* and *E. coli*. The PCR reaction conditions were optimized to permit detection of organisms from agar plates and fish tissues in less than 8 hrs. Each of the two pairs of oligonucleotide primers exclusively targeted 16S rRNA gene of the specific microorganism. When duplex PCR assay was used to simultaneous detection of the pathogens in asymptomatic fish, spleen and liver were negative for *A. hydrophila*, whereas kidney was positive for two bacteria. Samples of control group with negative results of duplex PCR were also negative by the culture method. On the whole, the duplex PCR has advantages in terms of its accuracy, sensitivity, ease of use, time of length analysis and cost-effectiveness compared to the single PCR and traditional method.

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Introduction

Fishes are often exposed to various microorganisms such as *Aeromonas hydrophila* that is naturally present in the fresh water environments (Belanco, 2000). This bacterium does not cause disease under optimal conditions, whereas under unfavorable environmental conditions, physiological stress or infection by other pathogens can cause motile aeromonad septicemia (MAS), epizootic ulcerative syndrome (EUS) and ulcerous dermatitis, which are led to economic losses in aquaculture industry (Plumb et al., 1976; Fang et al., 2000; Laptera et al., 2010). In addition, the presence of *Escherichia coli* in fishes is considered an indicator of the polluted or stressful environments where fish inhabit (DHSS, 1991; Gelderich et al., 1966; Sinderman, 1988). *Escherichia coli* enters aquatic ecosystems via animal excreta, agricultural runoff and human consumed wastes (Ferreira da silva et al., 2007; Berier et al., 2008). Therefore, significant numbers of *E. coli* on the skin and gut of the fishes can be led health risk to human (Janssen 1974; Ishii et al., 2007).

Rapid and accurate identification of the microorganisms, especially pathogen bacteria have a significant impact on fish health management programs (Adams et al., 2006). Traditionally, the diagnosis of the pathogenic bacteria is performed by

* Corresponding author: Alireza Mirvaghefi
E-mail address: avaghefi@ut.ac.ir

Table 1. Bacteria strains used in the experiment.

Bacteria species	Strain number	Donor
Escherichia coli	RTICC 2325	Razi Vaccine and Serum Research Institute
Aeromonas hydrophila	RTICC 1032	Razi Vaccine and Serum Research Institute

culturing bacteria on agar plates followed by phenotypic and serological properties (Altinok et al., 2008). However, detections of some bacteria are difficult due to their morphological variations and unusual biochemical reactions. Therefore, molecular diagnosis methods such as reverse transcription PCR, quantitative PCR, real-time PCR, AFLP, RFLP and RAPD have been developed to detect specific nucleic acids without culture and isolation of the pathogens (Tang et al., 2006; Adams et al., 2008).

In this regard, the individual PCR assay is an effective method for identification of the fish pathogens; however, a large number of individual PCR assays are necessary when a single primer set is used on a large number of the clinical samples, which can be a relatively costly and time-consuming process. Hence, the simultaneous detection of several pathogens with a multiplex PCR (m PCR) approach would be a relatively rapid and cost effective method (Mata et al., 2004). In this method, we seek to diagnose all possible pathogens, which can be occurred in each disease (Belak, 2007). The multiplex PCR assay for the simultaneous detection of fish pathogenic bacteria has been recently described (del Cerro et al., 2002; Mata et al., 2004; Altinok et al., 2008). Hence, in this work, a duplex PCR assay based on the 16s rRNA genes for the simultaneous detection of *A. hydrophila* and *E. coli* from pure cultures, and the challenged rainbow trout tissues (*Oncorhynchus mykiss*) including liver, kidney and spleen were performed and the results compared with single PCR assays for each bacterium.

Materials and methods

Fish: Fifty-two rainbow trout with a mean weight of 246 ± 20. 91 g (Mean ± SD) and mean length of 27.04 ± 0.90 cm (Mean ± SD) were obtained from a commercial fish farm in Karaj (Alborz Province, Iran). They were introduced at a rate of 13 fish per l000-liter to four tanks with proper aeration. Fish were acclimatized to the laboratory conditions for 2 weeks prior to experiment. The water temperature during acclimatization period and experiment was 14.5°C (± 1.5).

Bacteria: Bacterial isolates (*A. hydrophila* RTICC 1032 and *E. coli* RTICC 2325) were obtained from the Razi Vaccine and Serum Research Institute (Table 1). For the challenges, each bacterium strain was cultured on TSA (Himedia-M 290) at 37°C for 24 hrs and harvested in a sterile physiological saline to 10^{-9} and diluted to an 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 A. hydrophila and E. coli: For bacterial challenge, 9 fish (three from every tank) were randomly selected, anaesthetized in 100 mg/ml of Tricaine Methane Sulphonate (TMS) and injected intraperitoneally with 1 ml of the bacterial suspension of *A. hydrophila*, *E. coli* and mixture of *A. hydrophila* and *E. coli*, respectively. Then, injected fish were returned to treatment tanks and allowed to recover from the anesthetic. One of tanks was considered as controls group without injection.

Fish sampling: Forty-eight hours after injection, three fish from each treatment were sampled and their body surface were swabbed using 70% ethyl alcohol after killing by overdosing using TMS to prevent contamination from the rearing environment and normal external bacterial flora. To obtain similar size samples, 1 mm cubes of the liver, kidney and spleen were aseptically removed and put in the microcenterfuge tubes for detection of microorganism from fish tissue. Then, the samples of liver, kidney and spleen were streaked on tryptic soy agar. Following incubation, one typical colony was selected from each isolate and sub-cultured on

Table 1. Primers used in this study.

Name	Gene	Sequence (5-′3′)	Described	Pathogen	Size (bp)
FES RES	16s rRNA	F:GAAAGGTTGATGCCTAATACGA R:CGTGCTGGCAACAAAGGACAG	Nielsen *et al.*, 2006	*A. hydrophila*	700
FES RES	16s rRNA	F:GGAAGAAGCTTGCTTCTTTGCTG R:AGCCCGGGGATTTCACATCTGA	Sabat *et al*., 1999	E. *coli*	544

MacConkey (Merck-5465), EMB (OXOID-CM69) and Blood Agar media to check purity of the isolates. All isolates were stored in a broth culture supplemented with 15% glycerol at -70°C.

The isolates were classified as *A. hydrophila* or *E. coli* according to their reactions in the following conventional tests including catalase, motility, indole, voges-proskaues, urea, triple sugar iron (TSI), glucose, methyl red, H_2S production and citrate utilization tests based on Bergey's manual of determinative bacteriology (Holt, 2000). Every substrate was incubated at 37°C and reactions read after 24 and 48 hrs.

DNA extraction: For DNA extraction of the isolates (pure cultures), a boiled method was used based on Sambrook et al. (2001) by phenol-chloroform–isoamyl alcohol. DNA concentrations of samples were evaluated using a spectrophotometer. In addition, DNA was extracted form liver, kidney and spleen of artificially infected fish based on Altinok et al. (2008). The extracted DNA quality was evaluated using electrophorese on a 0.8% agarose gel.

Primers and PCR conditions: The used primers in this study were based on Nilsen et al. (2001) and Sabat et al. (1991) to validate the duplex PCR assay for the simultaneous detection of *A. hydrophila* and *E. coli* in asymptomatic carrier fish. The PCR protocol was optimized by amplification reaction in a thermal cycler (Astec, Japan) using the ready–to–go PCR beads (Cinagene, Iran). Reaction mixtures had 1 µL of each primer, 1 µL of the DNA template, 17.5 µL of sterile distilled water, 1 µL of $Mgcl_2$, 0.5 µL of dNTP, 2.5 µL of 10 x 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 template, (2) DNA from control fish and (3) DNA from *E. coli* and *A. hydrophyla* (Positive control). After the PCR, the products were transferred to a 1.5% agarose gel, electrophoresed, and DNA was visualized by ethidium bromide staining. Table 2 shows the sequences of the two primer pairs used in this study.

Duplex PCR assay: A duplex PCR assay was developed for the simultaneous detection of *A. hydrophila* and *E. coli* in which rainbow trout were experimentally challenged with both bacteria. The specificity and sensitivity of this assay was evaluated by performing the duplex PCR to the detection of healthy carriers. Samples were collected from kidney, liver and spleen and analyzed for the presence of these two pathogens by duplex PCR. To avoid contamination, each of the following steps were performed in a separate room: autopsy, DNA extraction, PCR master mix preparation, DNA quantification, addition to the PCR mixture, PCR reaction and electrophoresis. New disposable razor blades, forceps, and gloves were used for each fish to reduce potential contamination between fish.

Sequencing method: To verify that the specific primer-pair amplified *A. hydrophila* and *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 available at the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov).

Table 3. Identification and characterization of *E. coli* and *A. hydrophyla*.

Characteristic of isolates identified as *E. coli and A.hydrophyla* by both PCR and biochemical tests	**Biochemical tests**								**Literature (Millership, 1996) - *A. hydrophila***								**Literature (Forbes *et al.*, 1998) - *E. coli***								**Molecular diagnosis**
	Cit	TSI	Gas/ glucose	U	Cat	MR-VP	Nit	SIM	Gas/ glucose	U	Cat	MR-VP	Nit	SIM	Cit	TSI	Gas/ glucose	U	Cat	MR-VP	Nit	SIM	Cit	TSI	
Ah-1	+	A/A	+	-	+	++	+	+++	+	-	+	+ +	+	+ + +	+	A/A	- %10 +	-	+	+ -	+	-++	-	A/A K/A	700-bp
E-1	-	A/A	-	-	+	+ -	+	-++									Gas/ glucose	U	Cat	MR-VP	Nit	SIM	Cit	TSI	544 bp
Type strain RTICC 1032	-	A/A	+	-	+	+ +	+	+++									- %10 +	-	+	+ -	+	-++	-	A/A K/A	700-bp
Type strain RTICC 2325	-	A/A	-	-	+	+ -	+	-++									Gas/ glucose	U	Cat	MR-VP	Nit	SIM	Cit	TSI	544 bp
Urea =U, Catalase=Cat , Citrate =Cit, Gas from glucose =gas/g, Nitrate= Nit, Sulfide, Indole, Motility =SIM, MR-VP=Methyl red, Voges-proskau; TSI=Triple Sugar Iron																									

Figure 1. Fish injected with *A. hydrophyla* (note petechial at the fin based and tail rot).

Figure 2. Fish injected with *E. coli* (shows no clinical signs of disease).

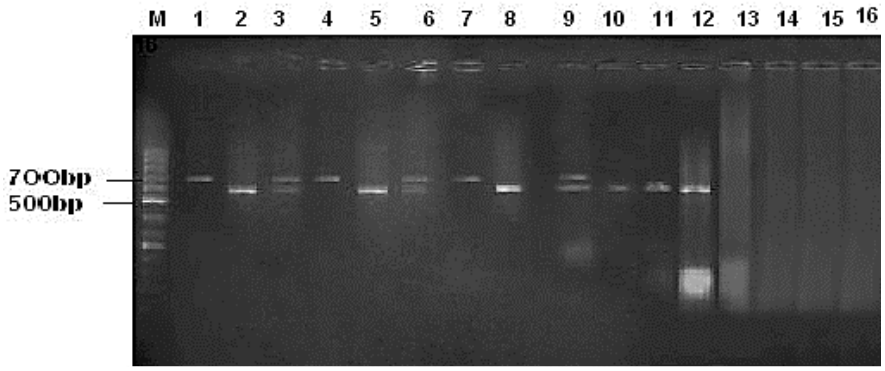

Figure 3. Representative PCR products from dead fish tissues, agar plate and positive control using the Duplex PCR and single PCR assay under optimized condition. Lane M, molecular size marker; lane 1, *A. hydrophila* RTICC 1032 (700bp); lane 2, *E. coli* RTICC 2325(544 bp); lane 3, mixture of the two bacteria; lanes 4 to 6, bacteria isolated from agar plates; lane 7, kidney tissue from fish injected with *A. hydrophila*; lane 8, kidney tissue from fish injected with *E. coli*; lane 9, kidney tissue from fish injected with two bacteria (asymptomatic fish); lane 10, liver tissue from fish injected with *E. coli*; lanes 11 to 12, liver and spleen tissue from fish were injected with two bacteria (asymptomatic fish), respectively; lanes 13 to15, negative control containing Duplex PCR from fish tissues (kidney, liver and spleen, respectively); lane16, no DNA.

Results

Isolation and identification of A. hydrophyla and E. coli: Pure cultures were obtained from all tissue samples and biochemical analysis were carried out on *A. hydrophyla* and *E. coli* isolates. All tissue samples were positive for microbiological and PCR identification (Table 3). Variation in citrate reaction was observed within the group identified as *A. hydrophyla* when compared to the type strain RTICC 1032.

Occurrence of A. hydrophila and disease signs: Mortality was observed two days post-injection in the group injected with *A. hydrophila*. *Aeromonas hydrophila* clinical signs were observed in five days post-injection including anorexia, exophtalmus, petechiae and reddening due to haemorrhage of the skin, erosion of the tail and fins and swimming at the surface of the tank (Fig. 1), and similar clinical signs were observed in the groups injected with both bacteria, while mortalities or clinical signs of disease were not observed in fish injected with *E. coli* (Fig. 2).

PCR identification of A. hydrophyla and E. coli: Three annealing temperatures (58, 60 and 62°C) and two $Mgcl_2$ concentrations (1.5 and 2 mM) were examined for the optimal sensitivity of the duplex PCR assay. A good intensity of the amplicons for each target DNA, as well as the absence of unspecific bands, was considered in selecting the optimal duplex PCR conditions. Thus, the best results were obtained with an annealing temperature of 58°C and 2 mM $Mgcl_2$. Each of the two pairs of oligonucleotide primers exclusively amplified the targeted gene of the specific microorganisms. Positive PCR amplification of DNA templates from *A. hydrophila* and *E. coli* were produced a single fragment of the expected, for each pathogen (700 bp and 544 bp, respectively) (Fig. 3). The two bacterial pathogens were simultaneously amplified with relatively equal DNA band intensities (Fig. 3).

Escherichia coli and *A. hydrophila* were detected from cultures on agar plates and fish tissues (Fig. 4). Detection of the two bacterial pathogens within DNA templates derived from liver, kidney and spleen were possible as early as 48 hrs after challenge in dead fish (Fig. 4A). The two bacterial pathogens were simultaneously amplified in kidney (Fig. 3); whereas, only *E. coli* was amplified in the liver and spleen tissues of fish injected with two bacteria. The size of PCR products from colonies were the same as tissues. Representative examples of the product formation from each source are shown in Figure 4. All tissue samples were positive for microbiological and PCR identification (Table 3). The total procedure was accomplished in less than 8 hrs. No amplification products were obtained from control group (Fig. 3). To confirm the positive PCR results, we sequenced the amplified DNA products

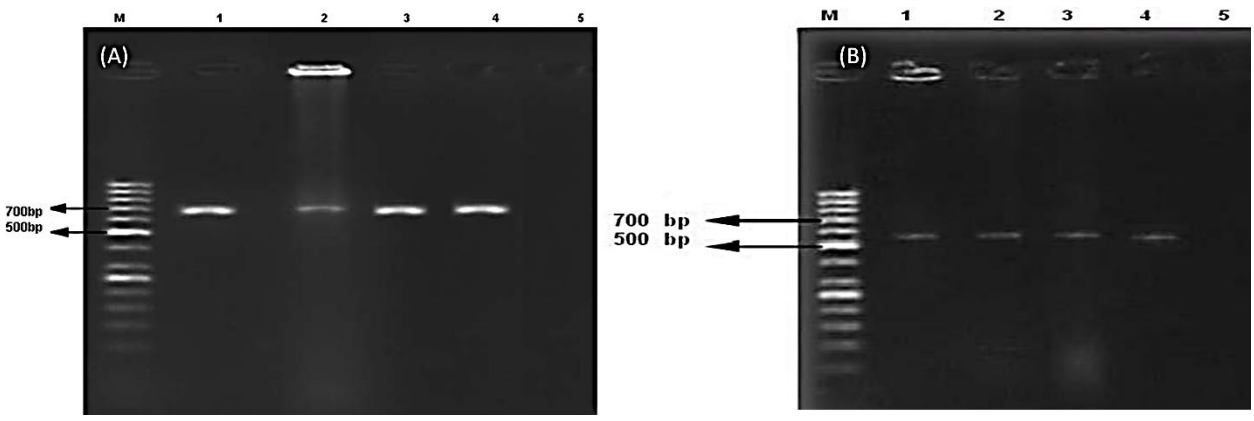

Figure 4. Specificity PCR products from dead fish tissues, agar plate and positive control using FAS-RAS primer set for detection of *A. hydrophila* (A). Lane M, molecular size marker; lane 1, *A. hydrophila* RTICC 1032 (700bp); lane 2, kidney; lane 3, spleen; lane 4, bacteria isolated from agar plate; lane 5, negative control (no DNA). (B) 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, negative control (no DNA).

from different PCR reactions and DNA extractions. The BLAST analysis from sequencing of four randomly amplicons showed similar results, and found that the amplified fragment exactly matched the sequence of two bacteria. These findings suggest the high specificity of the primers to detect *E. coli* and *A. hydrophila*.

Discussion

In aquaculture industry, diagnosis and treatment of the microbial diseases is crucial both from economic and sanitary point of views (Blanco et al., 2000; Stevenson, 1999). In recent years, there has been much interest in the development of multiplex PCR assay for the simultaneous detection of bacterial fish pathogens (del Cerro et al., 2002; Mata et al., 2004; Altinok et al., 2008).

Mortality was observed in 48 hrs post-injection in injected fish with *A. hydrophila*, showing a hemorrhagic septicemia with hemorrhagic in internal organs and a red tinged ascetic fluid. The kidney, liver and spleen of challenged fish were processed for both microbiological and single PCR analysis. In the literature, *A. hydrophila* is usually reported to be citrate positive (Millership, 1996). However, Neil and Nair (2004) reported that *A. hydrophila* to be citrate variable, which was confirmed in this study. This could be due to many biochemical identification schemes based on the analysis of human clinical isolates and that fish isolates may differ in several biochemical characters. Different reaction patterns may be influenced by physical parameters, such as pH, temperature, and growth substrate concentrations (Haenninen et al., 1994; Janda et al., 2002; Sautour et al., 2003). In this study, the characterization by biochemical identification methods was supported by the use of molecular technique.

Escherichia coli was detected 48 hrs after injection in the kidney and liver of the injected fish with this bacterium. Buras et al. (1987) reported that the peaks in the concentration of fecal coliforms in water can be detected after 2 weeks in the kidney and liver. These fish showed no clinical signs of disease. All tissue samples were positive for microbiological and PCR identification.

The results demonstrated that *A. hydrophila* and *E. coli* can be simultaneously detected in kidney from asymptomatic carrier fish and agar plates. In the duplex PCR assay, the amplification products corresponding to *A. hydrophila* (700 bp) and *E. coli* (544 bp) were obtained, which was supported by the sequencing results. The duplex PCR did not produce any non-specific amplification products. *Aeromonas hydrophila* was not isolated from liver and spleen. The high specificity of this assay was verified by the absence of amplified *A. hydrophila* DNA fragment in these samples.

The duplex PCR method described in the present work provides improved capabilities to detect *A. hydrophila* and *E. coli* in healthy carriers and also decreases the time required to amplify the 16SrRNA gene. Our results, based on the PCR suggested that the first target of *A. hydrophila* colonization is kidney of the rainbow trout showing the pathogen prior to clinical symptoms. Furthermore, our findings suggest that the combination of sampling method and duplex PCR is suitable for a rapid detection and discrimination between apparently healthy and asymptomatic infected and uninfected fish. Uninfected fish used as controls did not produce any amplification products with either method. Our study has confirmed that the usage of an individual PCR reaction for each pathogen and laboratory culture based methods is costly, tedious and time consuming. But the duplex PCR method could identify two bacterial pathogens in less than 8 hrs.

In conclusion, the duplex PCR presented in the current work is suitable tool for the detection of fish pathogen and at the same time, allows for rapid identification of bacterial causative agents of human diseases. These results, together with those obtained in dead fish and asymptomatic fish experiments, indicate that the kidney is suitable for epidemiological sampling. On the whole, the duplex PCR has advantages in terms of its accuracy, sensitivity, ease of use, time of length analysis and cost-effectiveness compared to the single PCR and traditional method.

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