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Short Communication

Detection of Pathogenic *Aeromonas hydrophila* from Rainbow Trout (*Oncorhynchus mykiss*) Farms in Turkey

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Abstract

The aim of this study was identifying and detecting virulent *Aeromonas hydrophila*, which caused bacterial hemorrhagic septicemia in rainbow trout (*Oncorhynchus mykiss*) farms in Muğla-Fethiye region by using PCR amplification of virulence-related genes. For this reason, clinically observed diseased fish samples were collected between 2009 and 2011. Three target genes, cytolytic enterotoxin (*AHCYTOEN*), hemolysin (*Hly*) and bacterial outer membrane protein (*OmpTS*) genes have been chosen for detecting virulent bacteria from those samples. Detected *A. hydrophila* from infected fish samples was demonstrated with positive amplifications of three genes at the same time. In addition, these positive results were separately confirmed by the amplification results of references strains. As a result, the infectious agent of this disease was identified rapidly by using the direct detection of bacteria through specific virulence determinants as genetic markers, before it spreads out.

Keywords: *Aeromonas hydrophila*; AHCYTOEN; Hly; OmpTS

Introduction

Aeromonas hydrophila is a gram negative, an opportunistic and zoonatically important primary fish pathogen which is the causative agent of bacterial hemorrhagic septicemia (motile aeromonad septicemia) (Austin and Austin, 1999; Chu and Lu, 2005). The disease is often associated with serious damage and economic losses in rainbow trout (*Oncorhynchus mykiss*) farming industry (Paniagua *et al*., 1990; Wang *et al*., 2003; Sağlam *et al*., 2006). *A. hydrophila* produces several extracellular products such as proteases, haemolysins, aerolysin, cytolytic enterotoxins that are related with its pathogenicity (virulence) (Kingombe *et al*., 1999; 2010; Hu *et al*., 2012).

Secreted extracellular hemolysin and cytolytic enterotoxin by bacteria are reported to be important for causing certain lytic activities in host cells (Watanabe *et al*., 2004; Uma *et al*., 2010). Additionaly, it has been shown that protein layers, O-antigens, fimbriae and outer membrane proteins of *A. hydrophila* play essential role of adherence of mechanism and contribute to colonization of fish tissue (Fang *et al*., 2004; Juarez *et al*., 2005; Khushiramani *et al*., 2007). The involvement of some virulence factors in *Aeromonas* spp. which were encoded by several genes, has been demostrated (Chacon *et al*., 2003; Xia *et al*., 2004). In the past, traditional microbiological and biochemical studies indicated that hemolytic and lytic activities were occured in virulent *A. hydrophila* strains (Kozaki *et al*., 1987; Santos *et al*., 1988). Moreover, despite the number of other studies performed on the house keeping genes to detect *A. hydrophila* such as 16 sRNA, entorotoxin (*act*), and DNA grase (*gyrB*), they were not able to confirm pathogenicity of bacteria (Ottaviani *et al*., 2011). Therefore, it is needed to developed more reliable, pathogen-specific and fast identification system to determine related virulence genes for identification of bacterial agent. With this method, not only fast identification can be done without much labor.

For this reason, we aimed rapid identification of only pathogenic strains of *A. hydrophila* from clinical isolates in rainbow trout in comparison to the reference strain by showing presence of three positive PCR-amplicons of hemolysin (Hly^+), cytolytic enterotoxin ($AHCYTOEN^+$) and bacterial outer membrane protein ($OmpTS^+$) gene determinants in the same sample.

Materials and Methods

Field Sample Collection

A total of 100 clinically observed infected fishes which ranged between 10-200 g from South-West Region of Turkey (Muğla-Fethiye) were collected for *A. hydrophila* between 2009 and 2011. Samples from skin ulcer, infected liver and kidney were taken by using sterile swabs. The ends of the swabs were cut off into both 15 mL falcon bottle containing 5 mL of transport medium (tryptic soy broth - TSB) and 5 mL of TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and then transferred to the laboratory for DNA extraction.

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Reference strain of *A. hydrophila* 19570 was obtained from American Type Culture Collection (ATCC), USA. Bacteria was grown in tryptic soy agar (TSA) plates and broths (TSB) at 22-25^{o}C for 24 to 48 h. Stock cultures were maintained in a broth medium supplemented with glycerol at -20^{o}C (Austin and Austin, 1999).

DNA Isolation

One mL of bacterial suspension from reference culture and swabs of field tissue were transferred to 2 mL centrifuge tubes, and centrifuged at 9000 x g for 5 min. Supernatants were discarded and the pellets were dissolved in 1 mL TE buffer (10 m*M* Tris-HCl, 1m*M* EDTA, pH 8.0). The genomic DNA was purified by using Qiagen DNA Extraction Kits (Qiagen, USA) as per the manufacturer's instructions.

Primers and PCR Amplification of Genes

Three pairs of primers (Table 1) were designed to detect targeted *AHCYTOEN*, *OmpTS* and *Hly* genes. The oligonucleotide primers were synthesized by Metabion (Germany).

PCR amplification of *AHCYTOEN* gene was performed by modifying the method of Kingombe *et al*. (1999) by using DNA thermal cycle (Techne, Inc., USA). The reaction mixture contained of 50-100 ng of template DNA, 0.25 µL of 0.5 U of Taq polymerase (Fermantas, USA), 5 µL PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl, 1.5 m*M* $MgCl_2$, pH 8.3), 250 nmol/L of each dNTP, 5 m*M* of each primers. PCR were run under the following conditions: heat denatutation at 94^{o}C for 2 min, primer annealing at 56^{o}C for 2 min and DNA extension at 72^{o}C for 1 min in each cycle. PCR of *OmpTS* gene was amplified according to the protocols of Khushiramani *et al*. (2007) and Juarez *et al*. (2005). PCR amplification for *Hly* gene was performed using a total volume of 50 µL of reaction mixture consisted of 5 µL template DNA, 1 U of Taq polymerase (Fermantas), 5 µL buffer (50 m*M* KCl, 10 m*M* Tris-HCl, 1.5 m*M* $MgCl_2$, pH 8.3), 4 µL of dNTPs (250 µmol/L of each), 500 nmol/L of each primers. PCR were run under the following conditions; preheating at 95^{o}C for 5 min followed by 30 cycles at 95^{o}C for 2 min, 55^{o}C 1 min and 72^{o}C for 1 min, followed by 7 min final extension at 72^{o}C.

All PCR products were analysed by gel electrophoresis 1% agarose (Applichem, USA) containing 0.6 µg/mL ethidium bromide in TE buffer. DNA bands were visualized over a UV transilluminator at 254 nm (DNR geldoc, USA).

Results

Total of 100 diseased fish samples were collected over the period of 2009 to 2011. In order to investigate whether those samples have been infected by pathogen *A. hydrophila* or not, direct PCR from their DNAs were amplified to show the targeted *AHCYTOEN*, *OmpTS* and *Hly* genes in <3 h.

DNA extract from direct field tissue samples and reference stain were subjected to PCR amplifications using specific primers (Table 1), which were designed to detect pathogen *A. hydrophila* by PCR. All primer sequences were compared against each other and homology searches performed against the GeneBank database for sequence similarities using BLAST program (NCBI, USA).

An amplimer of 232 bp for *AHCYTOEN* gene was obtained from both field and reference samples. Thypical positive amplicon results of *AHCYTOEN* gene in *A. hydrophila* are shown in Fig. 1. PCR products of 1008 bp of *OmpTS* gene were obtained from liver, kidney and skin samples and reference strains. The positive amplicons of *OmpTS* gene in *A. hydrophila* are shown in Fig. 2. An amplimer of 597 bp of *Hly* gene was obtained from both field and reference samples. Thypical positive amplicons of *Hly* gene to detect *A. hydrophila* are shown in Fig. 3. As a result, a total of 25 liver, 22 kidney and 21 skin lesion swabs samples were positive for all three virulence genes in *A. hydrophila*.

Discussion

Use of virulence-associated genes as detection markers is very convenient and rapid method to identify organisms without requiring traditional microbiological culturing process. Previous studies have shown that production of hemolysin, aerolysin, cytolytic toxins and bacterial membrane receptors might individually contribute the virulence of *A. hydrophila* (Kingombe *et al*., 1999; Gonzales-Serrano *et al*., 2002; Ottaviani *et al*., 2011). However, it is suggested that proteolytic, haemolytic and cytotoxic activities of *A. hydrophile* could be changed upon *in vitro* passaging and temperature changes during culturing process (Morgan *et al*., 1985). In the last decade, hemolysin or outer bacterial membrane protein gene encoding studies indicated that hemolytic and/or adhesin activities occurred in the presence of genes in virulent *A. hydrophila* strains (Gonzales-Serrano *et al*., 2002; Khushiramani *et al*., 2007).

In our study, six pairs of primers (Table 1) were generated and compared with other sequences in the GenBank database to display the ability of amplification efficiency. Our results confirmed that those primers were able to amplify three separate virulence genes in both reference strain and in direct liver, kidney and skin swabs in <3-4 h. We have shown that PCR amplification results of *AHCYTOEN*$^{+}$, *Hly*$^{+}$ and *OmpTS*$^{+}$ genes from each separate swab could indicate only virulent *A. hydrophila*. These genes were unable to detect non-pathogenic *A. hydrophila* strains, which did not contain the cytotoxin and hemolysine genes. Therefore, we demonstrated that 68 of 100 samples are virulent-related genes as a result of existence of hemolysin, adherence and enterotoxin activities. Like Wang *et al*. (2003), our results supported the view that bacteria isolated from health fishes did not carry the hemolysin genes.

Table 1: Primers used to detect pathogenic *A. hydrophila*

Primer	Sequence (5'-3')	Product lenght (bp)
AHCYTOEN -f	GAGAAGGTGACCACCAAGAACAA	232
AHCYTOEN -r	AACTGACATCGGCCTTGAACTC	
OmpTS-f	GCAGTGGTATATGACAAAGAC	1008
OmpTS-r	TTAGAAGTTGTATTGCAGGGC	
Hly-f	GGCCCGTAGCCCGAAGATGCAGG	597
Hly-r	CAGTCCAACCCACTT	

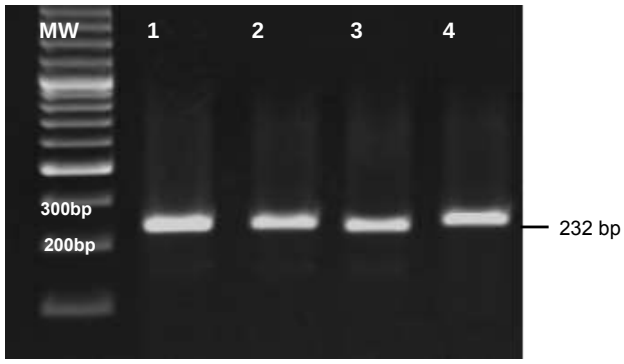

Fig. 1: PCR amplification products of *AHCYTOEN* genes (232 bp) from field and reference strain. MW. Molecular marker, 1. *AHCYTOEN*$^{+}$ gene from reference *A.hydrophila*, 2-3-4. *AHCYTOEN*$^{+}$ gene from liver, kidney and skin samples respectly

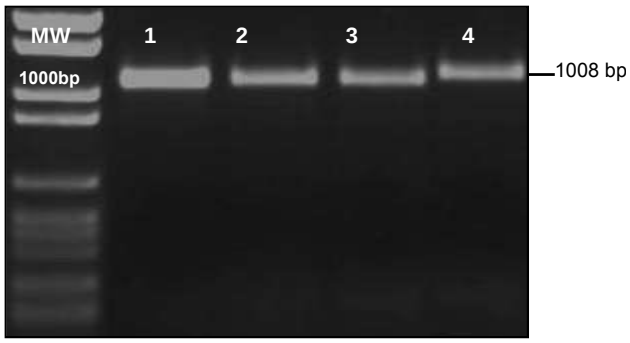

Fig. 2: PCR amplification products of *OmpTS* gene (1008 bp) from field and reference strain. MW. Molecular marker, 1. *OmpTS*+ gene from reference *A.hydrophila*, 2-3. *OmpTS*+ gene from kidney, liver and skin samples

Furthermore, the studies of Khushiramani *et al*. (2007) and Juarez *et al*. (2005) have confirmed our *OmpTS* gene amplification findings that virulent *A. hydrophila* has adherence activity to attach host epithelial cells surface mediated by presence of membrane proteins. Moreover, these proteins are known as potentially important to vaccine components (Juarez *et al.*, 2003). Fang *et al*. (2004) also showed the presence of major adhesion gene in virulent *A. hydrophila*. Lastly, Kingombe *et al.* (2010) showed the cytolitic enterotoxin gene in *Aeromonas* spp.

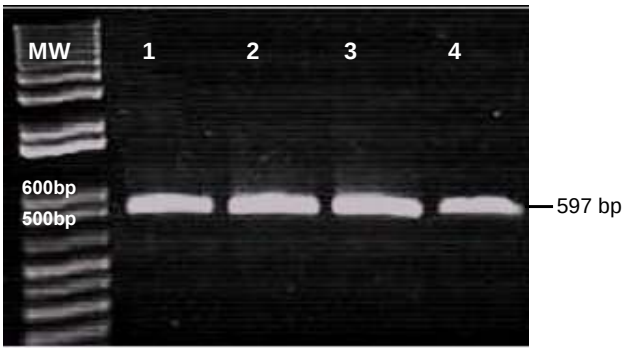

Fig. 3: PCR amplification of *Hly* gene (1008 bp) from field and reference strain. MW. Molecular marker, 1. *Hly*+ gene from reference *A.hydrophila*, 2-3-4. *Hly*+ gene from liver, kidney and skin samples respectly

In conclusion, we have developed rapid-multiplex gene markers method to detect only pathogenic *A. hydrophila*, which poses a threat to rainbow trout farms in Turkey. Further studies are needed to understand the pathogenesis of these bacteria and host relationship. The regional distribution of bacteria and analysis of nucleotide sequences in virulent isolates should also be studied to develop strain-specific DNA vaccine against this infection.

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