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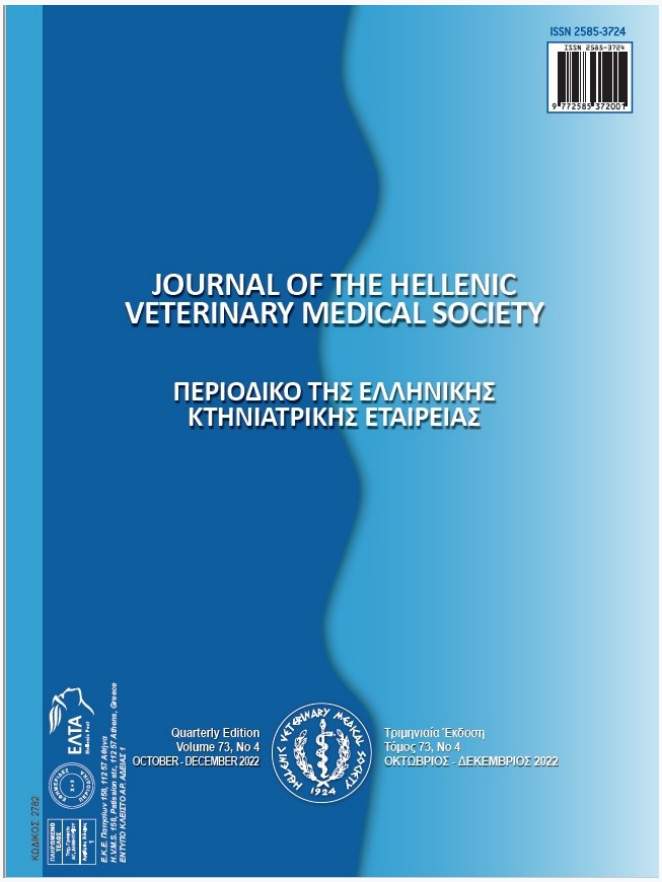

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Research article
Ερευνητικό άρθρο

Investigation of phenotypic and genotypic characteristics of isolated *Vibrio anguillarum* strains in rainbow trout cage farms in Kahramanmaras city of Turkey

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ABSTRACT. The purpose of this study is to isolate and examine the phenotypic and genotypic characteristics of the *Vibrio anguillarum* strains in rainbow trout cultured in floating cage farms in the province of Kahramanmaraş, Turkey. In June 2014, liver, spleen, kidney, and intestines samples of farmed rainbow trout (250-300 g) were collected from different fish farms in the region. Two methods were used to identify *V. anguillarum* in the samples. The Biolog System was used investigate the presence of *V. anguillarum* obtained from rainbow trout samples and examine the phenotypic and biochemical characteristics for the identification of *V. anguillarum*. The polymerase chain reaction technique with a specific primer was used to validate different *V. anguillarum* strains, according to phenotypic and biochemical characteristics. The results indicated that all of the isolated samples exhibited *V. anguillarum*. Sequence analysis was performed on the *V. anguillar*um strains obtained from the samples, and the corresponding data were analyzed. Compared to the culture method which required three weeks to identify *V. anguillarum*, the PCR technique provided rapid results in a matter of minutes.

Keywords: *Oncorhynchus mykiss*, Phenotypic, Genotypic, *Vibrio anguillarum*, BIOLOG GEN III, PCR, Sequence.

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INTRODUCTION

Listonella anguillarum, formerly known as *Vibrio anguillarum,* have been found and reported in 50 different fish species in 17 countries (Actis et al*.,* 2011; Austin and Austin, 2007).

The disease determined in *Sparus aurata* fishes in 1993 in Turkey (Candan, 1993; Çağırgan, 1993) was then reported in saltwater fishes such as *Dicentrarchus labrax* (Akan et al., 1996; Çağırgan, 2004; Korun and Timur, 2008; Tanrıkul et al., 2004), *Mugil cephlus* (Demircan and Candan, 2006), *Pagrus pagrus* (Korun and Gökoğlu, 2007), *Salmo salar* (Candan, 2000), and *Oncorhynchus mykiss* (Timur and Korun, 2004; Tanrıkul, 2007; Akayli et al., 2018).

Under stressful conditions, especially in conditions where the stocking is intense and water quality is poor, vibriosis causes serious economic loses in cultured fish. (Noga, 2000; Peggy and Francus-Floyd, 2002).

Investigated a practical, inexpensive, and effective vibriosis control a serious cause of severe mortalities of cultured European sea bass (*Dicentrarchus labrax*) using FKC bacterins of *Listonella anguillarum* or *Vibrio alginolyticus,* their cross-protection against *L. anguillarum* or *V. alginolyticus,* and their most suitable administration route in *D. labrax*. Experimental results indicated the superiority of *V. alginolyticus* FKC bacterin in protection against *V. alginolyticus* and cross-protection against *L. anguillarum* (Diab et al., 2021).

V. anguillarum strains obtained primarily from vibriosis outbreaks in Danish rainbow trout were sequenced, de novo assembled, and the genomes examined for the presence of plasmids, virulence, and acquired antibiotic resistance genes. The phylogenetic analysis and pan-genome calculations revealed great diversity within *V. anguillarum*. Serotype O1 strains were in general very similar, whereas considerable variation was found among serotype O2A strains (Hansen et al., 2020).

Vibriosis, caused by *V. anguillarum,* is a disease in which the mortality rate roughly exceeds 50% (Woo and Bruno, 2003). There has been no comprehensive investigation on the phenotypic and genotypic characteristics of *V. anguillarum*, which is the causative agent of Vibriosis that is a major fish disease in our country. In this study, besides the culture method used in the diagnosis of bacterial fish diseases, the Biolog System (The biolog GENIII micro plate) technique and molecular techniques were used to determine other phenotypic characteristics. In this way, the phenotypic and genotypic characteristics of *V. anguillarum*, which is an important bacterial disease in rainbow trout farming, was determined.

MATERIALS AND METHODS

This study was carried out in two different trout cage enterprises in the Kahramanmaraş province of Turkey. In June 2014, an anamnesis was taken from these enterprises and 20 fish specimens with disease suspicion and weighing 250-300 gr were brought to the laboratory.

All animal studies were approved by the Animal Ethics Committee of Kahramanmaraş Sütçü Imam University, Faculty of Agriculture (KSÜZİR-HADYEK) and Research Institute (Protocol number: 2013/3-1).

For microbiological isolation, the samples taken from the enterprises were incised abdominally from liver, spleen, kidney and intestines under aseptic conditions and were cultured in Brain Heart Infusion Agar and Tryptic Soy Agar. These media were incubated at 22°C for 48 hours. Pure colonies were obtained from the growing colonies in the media.

For microbiological identification, colony morphology, motion examination, Gram stain, potassium hydroxide, Catalase, Oxidase, oxidation/fermentation, Voges proskauer -Methyl red, and Indole tests were performed.

In this study, a standard micromed system, in which 94 biochemical tests were utilized for the identification of the Biolog GEN III Microplate gram-negative and gram-positive bacteria at the species level, was used.

Chromosomal DNA for propagation of genes to be cloned by using DNA isolation kit to isolate chromosomal DNA from *V. anguillarum* strain.

PCR was carried out in a total of 40 µL volume by using the Fermentas DNA polymerase enzyme. PCR mixture was prepared by adding 1 µL (20 picomol) forward specific primer empAF (5'-CAGGCTCGCAGTATTGTGC-3') and 1 µl (20 picomol) reverse primer empAR (5'-CGTCACCAGAATTCGCATC-3') (Xiao et al*.,* 2009). In the PCR process, DNA denaturation of 95°C as variable annealing, 72°C of extension temperature was set and the PCR

was completed in 25 cycles.

PCR products were run on agarose gel at concentrations ranging between 6% and 2%. After this, the agarose gel was dyed with ethidium bromide for 30 minutes, and then visualized and photographed under the UV light.

The DNA sequencing was performed with the ABI3130xl Gene Sequencing Device. Sequencing included the ExoSAP purification, Cycle PCR, and Sephadex purification steps. The DNA sequencing of the PCR products was carried out by using reverse and forward primers separately, and the sequences were analyzed with Chromas Pro and Clone Manager 9 program.

The obtained sequence results were edited and put in order in Clone Manager 9. Then, these sequence results placed in the NCBI GenBank on the electronic database and compared with the sequences of the other *V. anguillarum* species on the BLAST. The phylogenetic trees of the obtained species were drawn using the Mega 7 program.

RESULTS

Phenotypic and biochemical characteristics of 20 isolated *V. anguillarum* strains (Table 1) were determined from pure cultures.

In addition, Biolog System Device was used to validate biochemical tests and to determine other phenotypic characteristics (Table 2).

Table 1. Morphological and biochemical characteristics of 20 *V. anguillarum* isolated from Rainbow Trout.

Phenotypic and Biochemical Features	*V. anguillarum* (n:20)
Colony color	yellow
Gram Coloring	Red Gram (-)
Shape	comma
Oxidase	+
Catalase	+
Movement	+
H_2S	-
Methyl Red	-
Voges Proskauer	+
Indole	+
Urease Formation	-
Oxidation / Fermentation	F
MacConkey Agar	+
Mueller-Hinton Agar	+
Reproduction at 0°C	-
Reproduction at 5°C	+
Reproduction at 15°C	+
Reproduction at 20°C	+
Reproduction at 25°C	+
Reproduction at 30°C	+
Reproduction at 37°C	+
Reproduction in 0.0% NaCl	+
Reproduction in 0.5% NaCl	+
Reproduction in 1.0% NaCl	+
Reproduction in 2.0% NaCl	+
Reproduction in 6.5% NaCl	+

Table 2. Other phenotypic characteristics of 20 *V. anguillarum* isolated from Rainbow Trout (These characteristics were determined by Biolog System Device).

Biochemical criteria	Isolate reaction (n: 20)	Biochemical Criteria	Isolate reaction (n: 20)
pH 5	-	%8 NaCl	+/-
pH 6	+	%4 NaCl	+
Positive Control	+	%1 NaCl	+
Stachyose	-	N-Acetyl NeuraminicAcid	-
D- Turanose	Weak +	N-Acetyl-D-Galactosamine	+/-
Sucrose	+	N-Acetyl- β-D-Mannosa-mine	-
Gentiobiose	-	N-Acetyl-D-Glucosamine	+
D-Cellobiose	+/-	D-Salicin	+/-
D-Trehalose	Weak -	β- Methyl-D-Glucoside	+
D-Maltose	+	D-Melibiose	-
Dextrin	+	α-D-Lactose	-
Negative Control	-	D-Raffinose	-
D-Serine	+/-	Minocycline	-
Fusidic Acid	+	Rifamycin SV	+
%1 Sodium Lactate	+	Troleando-mycin	+
I Nosine	+	D-Serine	-
L-Rhamnose	-	D-Aspartic Acid	+/-
L-Fucose	-	D-Fructose-6- Phosphate	+
D-Fucose	+/-	D-Glucose-6- Phosphate	+
3-Methyl Glucose	-	Glycerol	+
D-Galactose	+	Myo-İnositol	Weak +
D-Fructose	+	D-Arabitol	-
D-Mannose	+	D-Mannitol	+
α-D-Glucose	+	D-Sorbitol	+
Niaproof 4	+	Tetrazolium Blue	+
Guanidine HCl	+	Tetrazolium Violet	+
Lincomycin	-	Vanco-mycin	+
L-Serine	+	D-Saccharic Acid	-
L-Pyroglutamic Acid	-	Quinic Acid	+/-
L-Histidine	+	Mucic Acid	-
L-Glutamic Acid	+	Glucoronamide	-
L-Aspartic Acid	+	D-Glucoronic Acid	-
L-Arginine	+	D-Gluconic Acid	+
L-Alanine	+	L-Galactonic Acid Lactone	+/-
Glycyl-L-Proline	+	D-Galacturonic Acid	+/-
Gelatin	+	Pectin	+
Potassium Tellurite	-	Sodium Bromate	-
Lithium Chloride	+	Sodium Butyrate	+/-
Nalidixic Acid	-	Aztreonam	+/-
Bromo-Succinic Acid	+	Formic Acid	+/-
L-Malic Acid	-	Acetic Acid	+
D-Malic Acid	+	Propionic Acid	-
α-Keto-Glutaric Acid	+/-	Acetoacetic Acid	+/-
Citric Acid	+	α-Keto- Butyric Acid	+
L-Lactic Acid	+	β- Hydroxy-D,L-Butyric Acid	-
D-Lactic Acid Methyl Ester	+/-	α-Hydroxybutyric Acid	Weak +
Methyl Pyruvate	+	γ-Amino-Butryric Acid	-
p-Hydroxy-Phenylacetic Acid	-	Tween 40	+

DNA isolation of 20 *V. anguillarum* isolates identified by biochemical tests and Biolog System tests was performed. These DNAs were amplified by the PCR. These products were then electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide and the results were evaluated under the ultraviolet transilluminator. In all twenty strains, 439 bp-long bands were seen with empAF and empAR primers belonging to *V. anguillarum* strain (Figure 1). Therefore, it was proven that all 20 isolates were *V. anguillarum.*

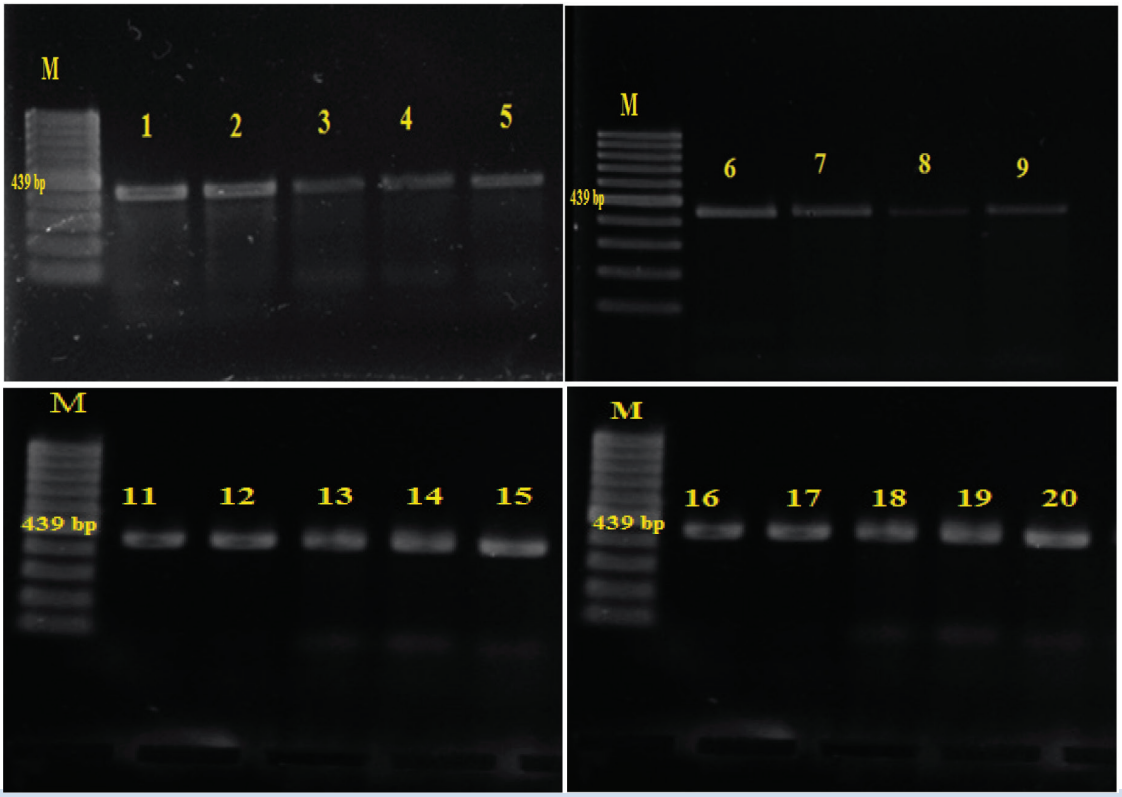

Figure 1. The PCR image of 20 samples.

Sequence analysis was performed by forward reading in 10 cases of *V. anguillarum* strains. The nucleotide sequence obtained from the sequence of 10 samples is shown in; the consensus sequence of *V. anguillarum* taken from NCBI is given in. These examples were aligned with the Clone manager 9 program and the result is shown in Figure 2.

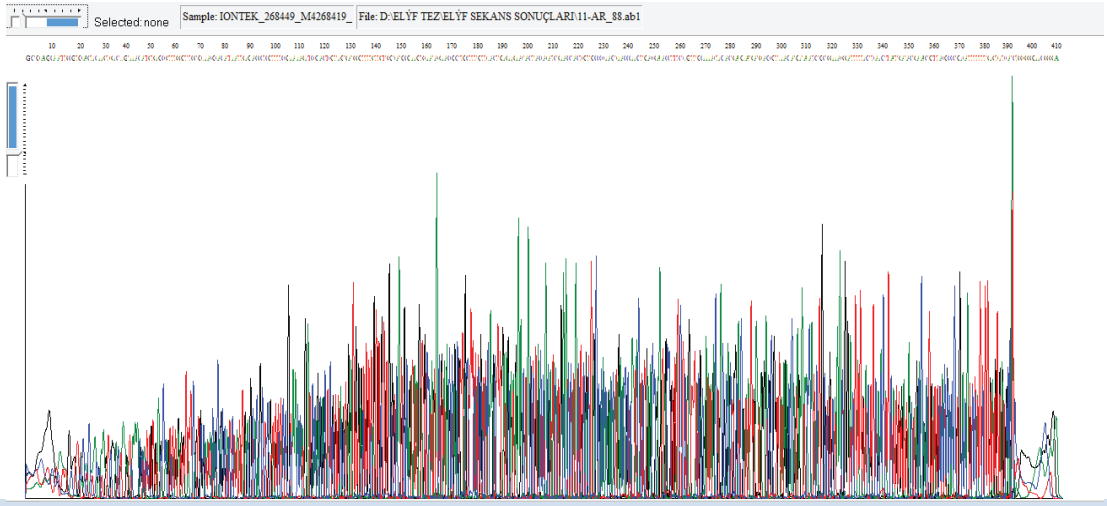

Figure 2. Alignment program of Clone manager 9.

The 16S gene region of 20 samples, identified as *V. anguillarum* species with phenotypic and genotypic characteristics, was amplified with empAF and empAR primers. In addition, by the obtained sequence results, it was confirmed molecularly that these samples were *V. anguillarum.* Moreover, the sequence results were compared with the reference *V. anguillarum* strains in NCBI in terms of similarity.

Using Mega 7 program, genetic similarities of isolates, which were identified as the same species by using sequences of about 439 base-pair length from the DNA region coding for 16S rRNA of *V. anguillarum* isolated from different regions as a result of the sequence study, were determined by the phylogenetic tree drawn by UPGMA Tree method. The phylogenetic tree is given in (Figure 3).

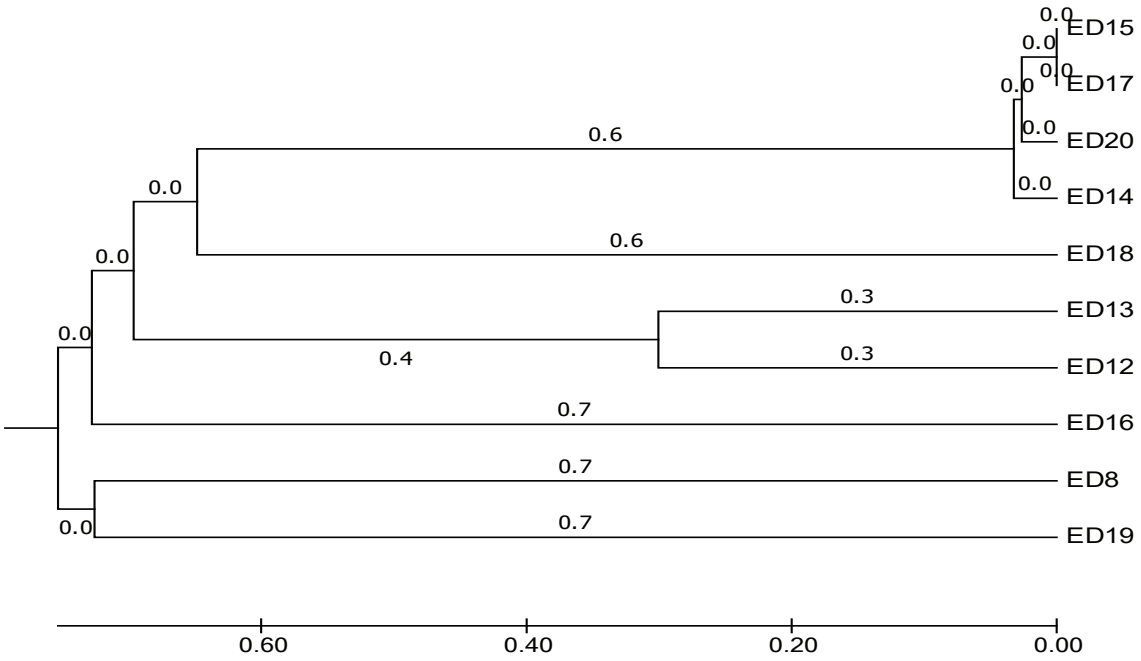

Figure 3. The phylogenetic tree, drawn based on the 16S rRNA gene region of *V. anguillarum* species

DISCUSSION

In this study, primarily, isolation and identification of *Vibrio anguilarum* from disease suspected trout of two different rainbow trout cage farms in the Kahramanmaraş province was carried out by the method known as classical culture method. Then, the sequence analysis of *V. anguillarum*, which was molecularly diagnosed by the PCR technique, was performed.

It has been stated that *V. anguillarum* produces more disease in sudden changes in water temperature (Akaylı, 2001; Akşit and Kum, 2008; Çağırgan, 1993; Demircan and Candan, 2006; Korun and Timur, 2008; Tanrıkul et al., 2004; Tanrıkul, 2007; Timur and Korun, 2004). In our study, *V. anguillarum* was isolated in June when there is seasonal change and water temperature change.

Vibriosis is seen as a major problem because of its widespread occurrence in the aquaculture world (Reed and Floyd-Francis, 2013; Toranzo and Barja, 1990). This disease is a bacterial infection that limits the production of marine fish, shellfish and arthropods (Akaylı 2001; Çağırgan, 1993; Demircan and Candan, 2006; Korun and Timur, 2008; Tanrıkul et al., 2004; Tanrıkul, 2007; Timur and Korun, 2004). Although *V. anguillarum* is considered to be the predominant species causing vibriosis, there are also some other vibrio species (*V. ordalii, V. damsela, V. vulnificus, V. chlerae, V. alginolyticus*) causing disease in many sea and hurd water species (Reed and Floyd-Francis, 2013). In fact, the disease spreads rapidly from plants to plants in intensive aquaculture areas (Tanrıkul, 2007). In rainbow trout plants in Kahramanmaraş, vibriosis has begun to appear and spread rapidly.

The disease usually begins with lethargy, loss of appetite, being near water surface or surface swimming, development of hemorrhages and occurrence

of swollen liver tissue. When it progresses, lowness on the skin and red and dead areas are formed, and the formed dots are spread around the fins and mouth (Arda et al., 2005). These clinical findings are in parallel with the findings obtained in our studies.

Vibriosis, caused by *V. anguillarum*, usually occurs in shallow and saline waters with high levels of organic matter in the summer months (Actis et al., 2011; Demircan and Candan, 2006; Eguchi et al., 2000; Frans et al., 2011; Hjeltnes and Roberts, 1993). In this study, isolation was performed in summer and in two sites where organic matter was abundant.

Morphological and necropsy findings are largely in line with the previous studies. They mostly isolated *V. anguillarum* during the seasonal transitions (Akşit and Kum, 2008). This suggests that the agent causes disease more in water temperature changes. In our study, also, observing of the disease and performing of the isolation occurred during the seasonal transition.

In this study, the identification test results (Table 1) of bacterial isolates were adapted to the previous knowledge of *V. anguillarum* (Akaylı et al., 2013).

Akaylı et al. (2013) found arginine, lysine, glucose and gelatin in the *V. anguillarum* isolates taken from rainbow trout. These findings are compatible with our study; however, arabinose is partially compatible. This situation can be interpreted as that the phenomenon of *V. anguillarum*, which causes disease in fresh water, may be phenotypically different from the factors in marine fishes, and a more comprehensive study is needed in this subject.

It has been stated that the PCR technique identifies a small number of microorganisms in pure or mixed cultures in a short period of time like one day, therefore, it is more advantageous than culture and serological tests (Lin and Tsen, 1996). The use of specific primers in the PCR amplification makes identification easier. In this study, the use of two different primer pairs of *V. anguillarum* spp. (empAF and empAR) eliminated the possibility of false positive results. Positive band formation in the samples obtained at the end of this study showed that *V. anguillarum* was the causative agent.

Sequence analysis was performed by the forward reading in 10 samples of *V. anguillarum* strains. The nucleotide sequence of *V. anguillarum* obtained from NCBI and the nucleotide sequence results obtained from the sequence analysis conducted in 10 samples were compared. These samples were aligned with the Clone manager 9 program and the phylogenetic tree was successfully determined.

Branch lengths in phylogenetic tree were 0.7 in ED8, ED16 and ED19 samples; 0.6 in ED18 and ED (14-15-17-20) samples; and 0.4 and 0.3 in ED12 and ED 13 samples, respectively.

CONCLUSIONS

In conclusion, *V. anguillarum* was isolated in all the samples obtained indicate that initiatives to stop the transmission of this pathogen are required. The risk of horizontal transmission of Vibriosis form a farm to a farm may be reduced by avoiding uncontrolled trade of fry and movement of fish. Furthermore, sterilization, and quarantine pools could be used combined with the usage of molecular techniques to rapidly and accurately screen the stocks for the presence of this pathogen.

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CONFLICT OF INTEREST

None declared.

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