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Research Article

Identification of *Vibrio anguillarum* by PCR (*rpoN* Gene) Associated with Vibriosis in Marine Fish in Turkey

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Abstract: The main causative agent of vibriosis is *Vibrio anguillarum*. In this study, 33 *V. anguillarum* strains were isolated from the internal organs of marine fish showing typical clinical signs of vibriosis, and these strains were identified by biochemical tests. For comparison of these results, API 20E and BIONOR Mono-Va agglutination kits were used. Finally strains were amplified by PCR using the *rpoN* gene. Although the phenotypic properties of *V. anguillarum* strains varied, the *rpoN* gene from chromosomal DNA was amplified in all strains. In conclusion, this gene could be used in the diagnosis of *V. anguillarum* strains isolated in Turkey.

Key Words: *Vibrio anguillarum*, PCR, *rpoN*, vibriosis

Türkiye'de Deniz Balıklarında Vibriozise Neden Olan *Vibrio anguillarum*' un PCR (*rpoN* geni) ile Tanısı

Özet: Vibriozis hastalığına neden olan etkenlerin başında *Vibrio anguillarum* gelir. Bu çalışmada, tipik vibriozis belirtisi gösteren deniz balıklarının iç organlarından 33 *Vibrio anguillarum* suşu izole edilmiştir ve bu suşlar biyokimyasal testlerle tanımlanmıştır. Sonuçların karşılaştırılması amacıyla, API 20E ve BIONOR Mono-Va aglütinasyon kitleri kullanılmıştır. En son PCR yoluyla suşların *rpoN* geni çoğaltılmıştır. *V. anguillarum* suşlarının fenotipik özellikleri çeşitlilik göstersede, kromozomal DNA' dan *rpoN* geni tüm suşlardan çoğaltılmıştır. Sonuç olarak bu çalışma, Türkiye'de izole edilen *V. anguillarum* suşlarının tanısında bu genin kullanılabileceğini göstermiştir.

Anahtar Sözcükler: *Vibrio anguillarum*, PCR, *rpoN*, vibriozis

Introduction

Vibriosis is a major disease occurring in marine and brackish water fish and characterized by hemorrhagic septicemia. The causative agent of vibriosis is the genus Vibrio, and the most important member of this genus, *Vibrio anguillarum*, causes disease in many fish such as the salmon, cod, char, halibut, Japanese eel, rainbow trout, and ayu, as well as shellfish such as the shrimp (1-10). Both phenotypically and serologically, *Vibrio anguillarum* strains show heterogeneity (7,10). Another bacterium belonging to the genus Vibrio, known in the past as *Vibrio anguillarum* biotype II and suggested by Schiewe et al. (11) as a new species, is *Vibrio ordalii*. Both species are closely related to each other and they cause epidemics of vibriosis, resulting in high mortality.

In recent years, especially under aquaculture conditions, infections caused by some Vibrio species other than *Vibrio anguillarum* and *Vibrio ordalii* have been reported in sea bass (*Dicentrarchus labrax*) vaccinated against vibriosis (N. Türk, Personal communication. Turkish Agricultural Ministry, Veterinary Research Institute of Bornova, İzmir, 2005). Therefore, a fast and effective diagnosis is extremely important in reducing fish mortality (7,12). Today, techniques such as polymerase chain reaction (PCR) and in situ hybridization are available for the diagnosis of pathogenic organisms (13) such as infectious pancreatic necrosis virus (14), *Myxobolus cerebralis* (15), *Piscirickettsia salmonis* (16), *Vibrio anguillarum* (17), and *Photobacterium damsela* subspecies *piscicida* (18). Recently, several genes of

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Vibrio anguillarum, such as hemolysin, *angE*, *rpoN,* and 16S rRNA genes, were cloned using PCR (17,19-21). In the present study, we used *rpoN* gene coding for the bacterial sigma factor (σ^{54}) to identify *V. anguillarium* in infected fish samples.

Sigma factor σ^{54} is responsible for regulating the genes providing coordination between carbon and nitrogen fixation in bacteria. This factor is also necessary for decarboxylic acid transportation, toluene and xylene catabolism, hydrogenase biosynthesis, and the translation of gene coding for flagella production and nitrogen fixation (22). O'Toole et al. (23) were the first group to sequence the 2218 bp *rpoN* gene. Then Gonzalez et al. (20) amplified the 519 bp portion of this gene to identify *Vibrio anguillarum* in fish blood and other tissues.

The aquaculture industry in Turkey suffers considerable economic losses due to diseases. Aside from preventive measures, difficulties in treatment in aquaculture operations and the lack of commercialized diagnostic tests for common fish diseases are some of the main concerns of the aquaculture industry in Turkey. Novel and sensitive diagnostic methods are therefore desperately needed to overcome these types of problem.

The aim of this study was to diagnose *Vibrio anguillarum* isolated from the internal organs of cultured (sea bass, *Dicentrarchus labrax*) and wild marine fish (mullet, *Mugil cephalus*) showing typical clinical signs of vibriosis by amplifying the *rpoN* gene using PCR. The diagnosis performed by PCR amplification was also confirmed with standard biochemical tests, API 20E kits and BIONOR Mono agglutination kits.

Materials and Methods

Fish and Isolation of Bacterial Strains

Eighteen samples were taken from 6 different fish farms in the Muğla region in May 2004. Moribund or newly dead sea bass and mullet were taken from cages. Fish samples with various clinical symptoms like hemorrhages on the fins, head, lateral line, around the eye, and operculum were brought to the laboratory. Bacterial strains were isolated from the liver, kidney, mouth and spleen of these fish. They were inoculated onto Tryptic Soya Agar (TSA, Oxoid) supplemented with 2% NaCl and incubated at 24 °C for 1-2 days. *V. anguillarum* IFO13266 reference strain (24) was also grown under the same conditions and used as a control. All strains and the control bacteria were examined biochemically. All strains were stored at –80 °C in nutrient broth with 20% glycerol.

Phenotypical Characteristics of the Bacterial Isolates

Biochemical characterization of all strains was performed by the following tests: Gram staining, motility, oxidase, resistance to O/129 (10 µg, 150 µg), growth on VAM (25), on thiosulfate citrate bile salt sucrose agar (TCBS, Merck) on TSA supplemented with 7% NaCl, glucose oxidation-fermentation test, nitrate reduction, β-galactosidase, arginine dihiydrolase, lysine decarboxylase, ornithine decarboxylase, Simmons' citrate, H_2S production, urease, tryptophan deaminase, indole, Methyl-red, Voges-Proskauer, gelatinase, acid from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, mellibiose, amygdaline and arabinose (26,27). Some of these tests were performed with API 20E strips (BioMérieux, France). The results were compared with Vibrios described by Austin and Austin (26). In addition to these tests, a BioNor Mono-Va agglutination kit (BIONOR, Norway) was also used for the identification of *Vibrio anguillarum*. The kit was used according to the manufacturer's recommendations.

DNA Isolation and Analysis

Chromosomal DNA from *V. anguillarum* was extracted by the method described in Ausubel et al. (28). Bacteria were grown in Tryptic Soy Broth (TSB) at 24 °C for 24 h and centrifuged. The pellet was resuspended in TE buffer (pH 8.0), and then lysed with 30 µl of 10% sodium dodecyl sulfate and 3 µl of proteinase K (20 mg/ml). After 1 h incubation, 80 µl of CTAB/NaCl solution was added. DNA was purified with an equal volume chloroform-isoamyl alcohol (24:1) followed by centrifugation at 14,500 rpm for 5 min. Further purification of the DNA in the supernatant was achieved by extracting with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Then isopropanol was added to precipitate the DNA. The purified DNA was pelleted by centrifugation at 14,500 rpm for 5 min and washed once with cold 70% ethanol before being dried in air. The dried DNA was resuspended in 25 µl of TE buffer and the DNA concentration determined with a Mikroquant spectrophotometer (Bio-tek Instruments Inc.) set at 260 nm wavelengths.

PCR Conditions

PCR amplification of the *rpoN* gene fragment was carried out with MBI Fermentas PCR mix according to manufacturer's recommendations. Primers (50 ng/µl) used in PCR reactions were designed related to Gonzalez et al. (20) as forward, *rpoN*-ang5' (5'-GTTCATAGCATCAATGAGGAG-3') and reverse, *rpoN*-ang3' (5'-GAGCAGACAATATGTTGGATG-3'). A DNA sample with the concentration of 100 ng was used as the template to amplify the *rpoN* gene using a Techne Progene thermal cycler. Thermocycling conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 40 s. A final extension step of 5 min at 72 °C was included. Then the samples were kept at 4 °C until analyzed.

Samples and 100 bp DNA ladder (MBI Fermentas) were loaded into 1.5% agarose gel prepared with 2 µg of ethidium bromide solution. The fragments were separated by horizontal gel electrophoresis (80 V, 30 min) in Tris-acetate-EDTA buffer (28), and visualized by UV transilluminator.

Results

Internal clinical signs of collected samples included hemorrhages in the gut and liver, enlarged spleen, and white mucoid material in the gut. In addition to these clinical signs, excessive fat and green colorization around the organs due to bile were observed.

All 33 strains isolated from fish samples and the reference strain grew on TSA including 2% NaCl morphologically in creamy color and round shaped colonies. Strains produced yellow colonies and haloes on TCBS and VAM. Gram negative, motile and slightly curved rod-shaped strains were cytochrome oxidase production test positive. The rest of the phenotypic characteristics are listed in the Table.

Isolated *Vibrio* strains were differentiated from *Vibrio ordalii* by their ability to dehydrolyze arginin, acid production from sorbitol, growth at 37 °C, and positive result from ONPG and Voges-Proskauer. We found the same results with API 20E rapid identification kits. Based on these findings, they were identified as *Vibrio anguillarum* strains. Furthermore, isolated strains gave positive results with BIONOR Mono-Va Agglutination kits.

Table. Phenotypic results of *Vibrio anguillarum* strains.

Tests	*V. anguillarum* Strains
ONPG (o-nitrophenyl b-D-galactopyranoside)	+*
Arginine dihydrolase	+
Lysine decarboxylase	–**
Ornithine decarboxylase	–
Simmons' citrate	+ (32/34)***
Catalase	+
Indole	+ (30/34)
OF	Fermentative
H_2S production	–
Urease	–
Nitrate reduction	+ (30/34)
Gelatinase	+
Methyl Red	–
Voges–Proskauer	+
Acid production from:	
Glucose	+
Mannitol	+ (23/34)
Sorbitol	+
Saccarose	+
Arabinose	+ (20/34)
Growth at:	
37 °C	+
0.5% NaCl	+
7% NaCl	+
Growth on:	
TCBS	+
VAM	+
O/129 (150 µg)	+
O/129 (10 µg)	+ (31/34)
API 20E results	++--+---+++++-+-+---+

*+: Positive, **–: Negative, *** Numbers in parentheses are the strains that gave written results.

Amplification of the *rpoN* gene with primers described in the Materials and Methods yielded bands approximately sized 519 bp. All strains showed 519 bp amplification product and were identified as *V. anguillarum*. The Figure shows 8 randomly selected samples of all PCR products on the 1.5 % agarose gel.

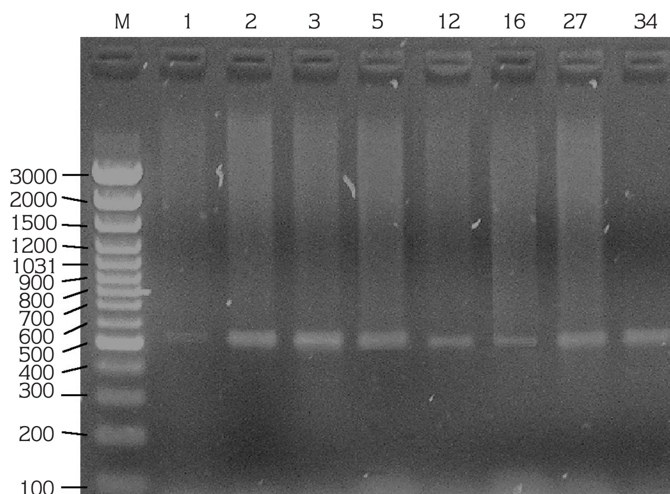

Figure. 519 bp bands on 1.5% agarose gel. The first well is marker, and the other wells are *Vibrio anguillarum* strains. Number 34 is IFO 13266 reference strain.

Discussion

Vibrio anguillarum was isolated from fish samples showing typical clinical and autopsy findings: externally, hemorrhages on the head, lateral line, around the eyes, inside the mouth, at the base of the fins, and around the anal region; internally, hemorrhages on the intestine wall, liver; hypertrophic spleen, and presence of white-opaque mucous substance in the intestine. This clinical table shows similarity to the findings reported by other research groups (2,4,7,10,26). Phenotypic characteristics of *Vibrio anguillarum* have been previously published (5-7, 11,26,29). Our findings on biochemical characteristics are similar to the findings described in the literature.

As reported by Benediktsdóttir et al. (6), Actis et al. (7), and Austin and Austin (26), 33 Vibrio strains have been described as *Vibrio anguillarum* and reported as different from *Vibrio ordalii* based on the Voges-Proskauer test, acid formation from sorbitol, and positive growth at 37 °C. Additionally, with these 33 strains, these researchers observed variable results from Simmons' citrate utilization, indole production, and acid formation by arabinose and mannitol. In a different study, Schiewe et al. (11) also noted some biochemical variations among *V. anguillarum* strains.

In addition to these biochemical tests, API 20E and BIONOR Mono-Va agglutination kits were used for rapid confirmation. API 20E kits gave similar results as mentioned in Austin and Austin (26). The BIONOR Mono-Va agglutination kit found useful diagnostics for rapid identification. However, to work with this kit, bacteria must first be isolated and then purified. We obtained positive results from this kit, as did Romalde et al. (30).

Diagnosis of *Vibrio anguillarium* using biochemical methods is difficult due to this phenotypic difference. Alsina et al. (25) reported that *V. anguillarum* strains grow better in VAM selective medium developed by this group than in TCBS. Based on their results, *V. anguillarum* strains grew both in VAM and TCBS media, but VAM medium gave quicker results. These heterogeneities in biochemical testing mentioned above necessitated the improvement of disease diagnostics in bacterial fish diseases. PCR and other molecular diagnostic tools readily became available for the scientific community recently.

Gonzalez et al. (20) diagnosed *V. anguillarum* strains from fish blood and tissue samples by amplifying the 519 bp portion of the *rpoN* gene using PCR. In our study, by using the same portion of this gene, diagnosis of *V. anguillarum* was performed using Vibrio strains directly isolated from the internal organs of fish. Amplified PCR products showed the amplification of 519 bp *rpoN* gene and resulted in the successful diagnosis of *V. anguillarum.* Our findings were in accordance with Gonzalez et al. (20), and the positive results obtained from all samples using this gene showed that this gene could be used in the diagnosis of *V. anguillarum* strains isolated in Turkey.

Finally, for the first time in Turkey, *V. anguillarum,* the causative agent of vibriosis in marine fish and especially in sea bass grown under aquaculture conditions, was diagnosed with PCR. These results eliminated the heterogeneity caused by the use of standard biochemical tests such as API 20E and BIONOR Mono agglutination kits in diagnosis and proved the importance of using vaccines containing increased numbers of strains for the vaccination of cultured sea bass.

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