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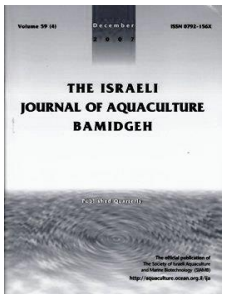

New rRNA primers for the detection of *Vibrio anguillarum*

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Abstract

In this work, 51 *V. anguillarum* isolates (42/51 of O1 serotype, 9/51 of O2) obtained from cultivated marine fish by Avsever and Ün (2015) were used. These isolates were confirmed with universal primers specific to *amiB* genes. They were also detected successfully at a rate of 100% with a new primer couple designed according to 16S ribosomal RNA sequence. The new primers were found to have a high sensitivity and specificity but were liable to give a cross-reaction with *Vibrio ordalii.*

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Introduction

Vibriosis caused by *Vibrio anguillarum,* which has been reported in 50 different fish species in 17 countries, is the most important bacterial fish disease (Austin and Austin 2012, Actis et al. 2011). *V.anguillarum,* unlike other *Vibrio* spp. which are mostly opportunistic, is a primary pathogen which causes fatal hemorrhagic septicemia in many fish species, crustaceans, and bi-valve mollusks (Toranzo et al. 2005).

Classical isolation is the gold standard for *V. anguillarum* diagnosis as well as many other bacterial and viral disease agents. Isolation is generally followed by identification procedures in which various biochemical tests are performed. Still, this procedure is time consuming and inefficient (Austin and Austin 2012). On the other hand, API 20E System, BioLOG fingerprinting method and Biotype-100 systems may lead to misidentification (Grisez et al. 1991, Kuhn et al. 1996, Austin et al. 2012), and VITEK 2-Compact identification system can occasionally be incompatible for fish pathogens as its incubator is set at 35.5^{o}C while bacterial agents in fish are generally mesophilic.

For this reason, molecular procedures have become increasingly popular. Plasmid analysis, ribotyping, pulsed field gel electrophoresis, and DNA hybridization methods may be adopted for this purpose (Skov et al. 1995, Martinez-Picado et al. 1996, Austin et al. 2012, Thompson et al. 2004). However, Polymerase Chain Reaction (PCR) is the most popular method as it is rapid, cost-efficient and easy to use (Hirono et al. 1996, Gonzalez et al. 2004).

In the PCR identification of *V. anguillarum,* various reports targeted specific gene regions for amplification. Species specific gene regions such as *amiB* (Hong et al. 2007), *rpoN* (Demircan and Candan 2006), *groESL* (Kim et al. 2012), *empA* (Xiao et al. 2009), *rpoS* (Kim et al. 2008), *recA* (Dorsch et al. 1992) and 16S rRNA (Kita-Tsukamoto et al. 1993) were among them. Within these target genes the method utilizing primers based on the *amiB* species specific gene region was reported to be superior to others. These methods could not differentiate between *V. anguillarum* and *Vibrio ordalii,* whereas *amiB* specific gene region amplification method was found to cause no cross-reactions with the other 25 members of the *Vibrio* genus (Hong *et al.* 2007).

Primer couples used in the identification of *V. anguillarum* seem to solve the problems regarding the diagnosis of Vibriosis cases. However, standardization of new primer couples targeting the causal agent contribute in terms of authenticity as they enable researchers to use phylogenetic analysis of new gene regions as well as providing new opportunities in disease diagnosis.

In this study we aimed at diagnosis with a new primer couple on *V. anguillarum* isolates which were previously identified with *amiB* gene specific universal primers and investigation of the sensitivity and specificity of these new primers.

Materials and Methods

Bacteria. In this study, 51 *V. anguillarum* isolates (MC1-51, 42/51 were O1 serotype, 9/51 were O2) obtained from cultured marine fish, and identified/confirmed with conventional microbiological methods and *amiB* gene specific universal primers by Avsever and Ün (2015) were used. Isolates were obtained from 6 different locations in the Aegean region (Milas, Dikili, Urla, Çeşme, Karaburun, Didim), and from 5 different fish species (sea bass, sea bream, sharpsnout sea bream, meagre and turbot). The strains used to investigate sensitivity of primers were taken from the Culture Collection of the laboratory (Fish Diseases NRL, Turkey). These were; *Vibrio algynoliticus* ATCC 17749, *Vibrio parahaemolyticus* CRL 1902, *Vibrio furnissii* ATCC 1128, *Vibrio vulnificus* ATCC27562, *Vibrio metschnikovii* (field strain, ML1,), *Vibrio splendidus* (field strain ML 14), *Vibrio fluvialis* (field strain, ML 9), *Photobacterium damsale* sp.(subsp.) *piscicida* ATCC 51736, *Pseudomonas flourescens* ATCC 49642, *Aeromonas hydrophila* ATCC 7966, *Tenacibaculum maritimum* ATCC 43398 and *Flavobacterium psycrophilum* ATCC 49511.

Design of new primers. Primers based on the coding region of 16S rRNA of *V. anguillarum* were designed using primer design software (primer 3). This primer couple was confirmed to be specific and original by controls of relevant scientific literature and they were produced commercially. The newly synthesized primers were vacl F (5'-GTGAGGTAATGGCTCACCAAG-3'), and vacl R (5'CTCTGGATGTCAAGAGTAGGTAAGGT-3').

DNA extraction and PCR amplification. DNA extraction was performed on 51 *V. anguillarum* isolates with High Pure PCR Template Preparation Kit (Roche, France, Lot: 11054300) according to manufacturer's instructions. Annealing temperature for the new primers was calculated with the Formula 4 (G+ C) + 2(A+ T). 749 bp. gene region on 16S r RNA of the 51 *V. anguillarum* isolates were amplified. Master mix consisted of Taq DNA polymerase with 1.25 U enzyme (0.6 µl), 10X Taq buffer minus reagents (2.5 µl), 25 mM $MgCl_2$ (2.5 µl), dNTP mix (0.2 mM) (2.5 µl), *vacl* F and *vacl* R primer mix (10 pM) (0.6 µl), DNA template (5 µl, 100 ng), and nuclease-free water. Total reaction volume was 25 µl with 11.30 µl master mix. Reaction parameters in the Thermal Cycler (Techne, TC-412) were 95 ^{o}C for 10 min. pre-denaturation, followed by 30 cycles of denaturation at 95 ^{o}C for 1 min, annealing at 65 ^{o}C for 1 min., and extension at 72 ^{o}C for 1 min. Final extension was 72 ^{0}C for 7 min. PCR products were subjected to electrophoresis in 1.5 % agarose gel with 0.2 ug/ul ethidium bromide within 1x TBE under 100 V (Volt) electrical currency for 60 minutes (Thermo, Primo ^{TM}). Bands were visualized with designated equipment (Vilber Lourmant, E-BOX VX5).

Sequencing of 16S rDNA. The PCR products sampled (6 of 51) according to location (Didim, MC10, O2 serotype; Milas, MC1, O1; Dikili, MC48, O2; Urla, MC44, O1; Çeşme, MC37, O2; Karaburun, MC25, O1) were submitted for sequence analysis. Sequencing was carried out in Macrogen Cooperation (Netherlands). Purified sequencing reaction mixtures were automatically electrophoresed using an Applied Biosystems model 3130 x l automatic DNA sequencer. The final sequence of the PCR fragments was determined from overlapping sequence data using SeqScape software (Applied Biosystems). One of the sequencing results was manually aligned to GenBank database.

Specificity and sensitivity of the primers. To investigate the specificity, 51 *V. anguillarum* strains were used with *Vibrio anguillarum* ATCC 19264, *Vibrio algynoliticus* ATCC 17749, *Vibrio parahaemolyticus* CRL 1902, *Vibrio furnissii* ATCC 1128, *Vibrio vulnificus* ATCC27562, *Vibrio metschnikovii* (field strain, ML1,), *Vibrio splendidus* (field strain ML 14), *Vibrio fluvialis* (field strain, ML 9), *Photobacterium damsale* sp. (subsp.) *piscicida* ATCC 51736, *Pseudomonas flourescens* ATCC 49642, *Aeromonas hydrophila* ATCC 7966, *Tenacibaculum maritimum* ATCC 43398 and *Flavobacterium psycrophilum* ATCC 49511. To investigate of the sensitivity, purified DNA of *Vibrio anguillarum* ATCC 19264 was diluted to 100 ng. Two-fold dilutions were then prepared (100 ng= 7.38 x 10^4 CFU, 50 ng =3.69x 10^4 CFU, 25 ng=1.84x 10^4 CFU, 12.5 ng= 0.92 x10^4 CFU, 6.25 ng= 0.46 x 10^4 CFU), and detection limit for DNA was established.

Results

New primers were observed to confirm 51 *V. anguillarum* isolates (42/51 were O1 serotype, 9/51 were O2) accurately (100 %) and no cross-reactions with other strains (Fig. 1) were found. The DNA detection limit was 12.5 ng (0.92 x10^4 CFU) (Fig. 2).

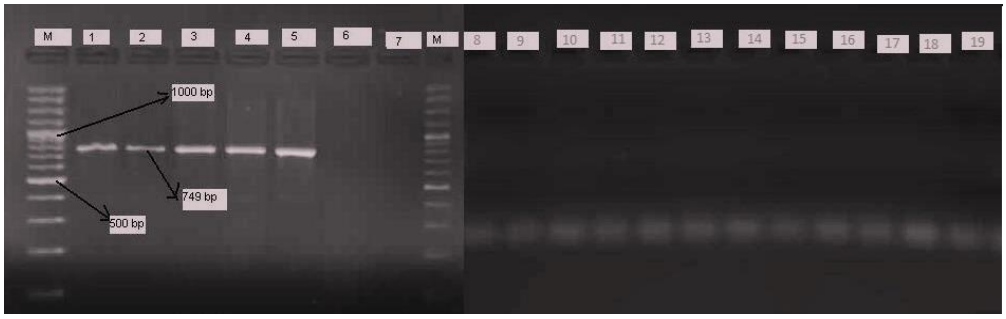

Fig. 1. Gel electrophoresis displaying the detection of 51 *V. anguillarum* isolates with new primer couples and lack of cross reactions with others M: 100 bp marker, 1: *Vibrio anguillarum* ATCC 19264 positive control, 749 bp, 2-5: *Vibrio anguillarum* isolates (MC 1, 2, 3, 4), 749 bp, 6: *Vibrio alginolyticus* ATCC 17749 negative control, 7: Negative control, distilled water, 8: *Vibrio parahaemolyticus* CRL 1902, 9: *Vibrio furnissii* ATCC 1128, 10: *Vibrio vulnificus* ATCC27562, 11: *Vibrio metschnikovii* (field strain, ML 1) 12: *Vibrio splendidus* (field strain, ML 14), 13: *Vibrio fluvialis* (field strain, Ml 9), 14: *Photobacterium damsale* sp. *piscicida* ATCC 51736, 15: *Pseudomonas flourescens* ATCC 49642, 16: *Aeromonas hydrophila* ATCC 7966, 17: *Tenacibaculum maritimum* ATCC 43398, 18: *Flavobacterium psycrophilum* ATCC 49511, 19: *Yersinia ruckerii* ATCC 29473.

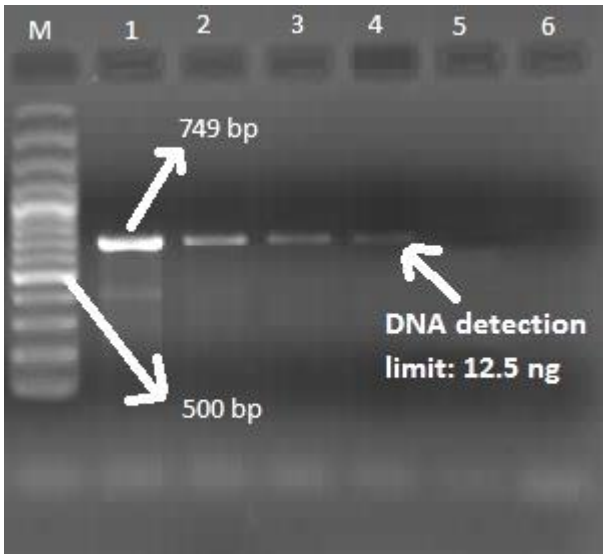

Fig. 2. Detection limit for DNA M: 100 bp, marker; 1-5: Varying DNA concentrations; 1: 100 ng, 2: 50 ng, 3: 25 ng, 4: 12.5 ng (DNA detection limit), 5: 6.25 ng, 6: Negative control, distilled water.

From this pool of PCR products, 6 samples (MC1, 25, 37, 44, 48, 50) were taken to be representative for each location and submitted to sequence analysis. The sequence result of the PCR amplicon of MC1 was determined and deposited in the GenBank database under the accession number KR817812. Phylogenetic relationship between the isolates was investigated using the 682 bp. region in the nucleotide sequence. Homology between isolates was observed and they were found to be 100% identical with the sequencing results of *V. anguillarum* isolates (Genbank Accession number KF460456.1, KF150778.1, AB680389.1) from previous reports. Cross reaction with *V. ordalii* (AB497069.1) was noted (Fig. 3).

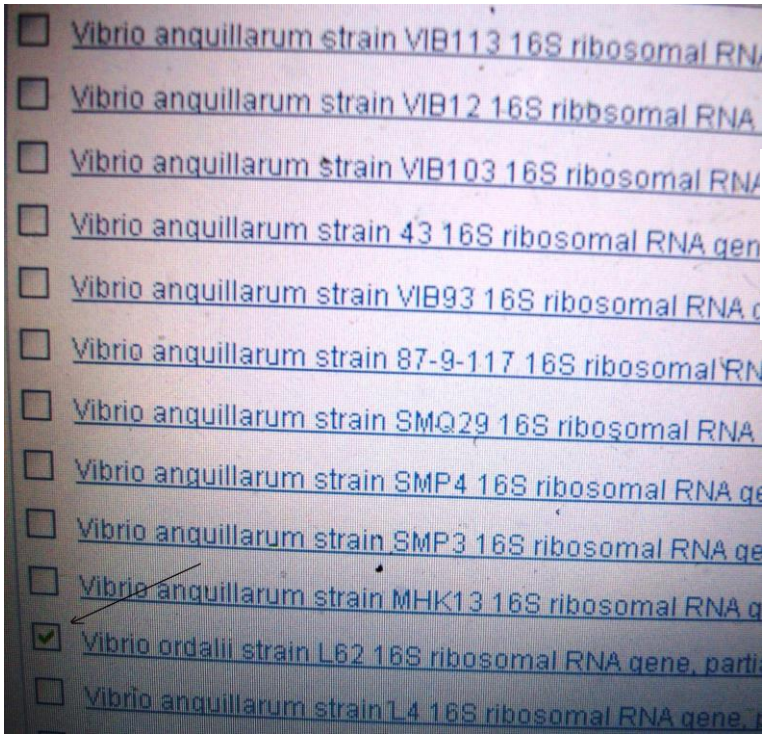

Fig. 3. Compatibility of isolates with others (100%) and cross-reaction of the amplified region with originally designed primers with *V. ordalii.*

Discussion

Many primer couples have been reported for *V. anguillarum.* But the sensitivity and specificity rates of these primers may be low. Also there may be errors or lack of reliability in primer sequences. Thus, it is a recommended solution for researchers to design their own primers. However, the sensitivity and specificity of newly designed primers should be established.

As primers designed in this study can detect 51 *V. anguillarum* (42/51 were O1 serotype, 9/51 were O2) at a rate of 100% accuracy without any cross-reactions with other strains, they can be considered to be suitable for *V. anguillarum* identification. Still, this primer couple may not be suitable for differentiating *V. anguillarum* from *V. ordalii* which is genetically the closest. Although this situation is the greatest disadvantage in many primer couples designed to identify *V. anguillarum*, it is still tolerable as the phentoypic characteristics and clinical symptoms caused by *V. anguillarum* and *V. ordalii* are easily discernable (Austin and Austin, 2012).

The reason for choosing an rRNA coding gene region for primer design is the frequent utilization of this region for phylogenetic studies. The lack of any difference of nucleotides between isolates from different locations may be due to conservation of this gene region. Also, it can be deduced that primers have targeted a highly stable gene region.

DNA detection limit for these primers was observed to be 12.5 ng (0.92 $x10^4$ CFU). This was similar to the results obtained in other reports. Gonzalez et al. (2003) found ($2x10^3$–$2x10^4$ cells/g) in fish tissue; Hong et al. (2007) found ($8x10^2$ CFU/g) in infected flounder tissue while Xiao et al (2009) found (3.3x 10^2 CFU/mL) in pure cultures of *V. anguillarum* M3, and (4.1x 10^2 CFU/g) in turbot kidney homogenates.

As a result, it was concluded that the primers designed in this work can be used for the diagnosis of *V. anguillarum* with high sensitivity and specificity rate. Cross-reaction with *V. ordalii* is possible. For this reason, simple phenotypic tests are recommended to differentiate between *V. anguillarum* and *V. ordalii* when using these primers for diagnostic purposes. However, new primer couples have been found relevant as they assist researchers in the phylogenetic analysis of new gene regions as well as contributing to disease diagnosis.

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