Agent-Specific Primer and Probe Design Using Bioinformatic Methods in Bacterial Fish Diseases

Şükrü Önalan*
Van Yüzüncü Yıl University, Faculty of Fisheries, Department of Diseases
PO box 65080, Van, Turkey
E-mail: sukruonalan@yyu.edu.tr

Mahmut Tüncer
Van Yüzüncü Yıl University, Faculty of Fisheries, Department of Diseases
PO box 65080, Van, Turkey
E-mail: tuncermahmut@hotmail.com

Mustafa Çevik
Van Yüzüncü Yıl University, Faculty of Fisheries, Department of Diseases
PO box 65080, Van, Turkey
E-mail: mustafacevik155@hotmail.com

Abstract
The incidence of diseases in fishes grown in parallel with the development of aquaculture in our country is also increasing. Early isolation and identification of the disease is important for the treatment process. Bioinformatics software is invaluable in terms of both the specificity of the study design and the minimization of errors that can be faced, in order to construct a molecular-based work in the field of aquatic diseases.In this study, it is aimed to design the study by using specific Bioinformatic software and specific primer and probe in molecular based studies to be carried out with bacterial fish disease agents. For this purpose, *Listonella (Vibrio) anguillarum* sample which is effective in Listonellozis.In the results of working; On the genome with the accession number NCBI LK021130.1, the genes in the homology-low regions were screened. Probing experiments were carried out by selecting genes that contain the most appropriate specific sites for primer insertion. The vabS gene has been found to have the most appropriate sequence for this purpose. Specific areas for the primer on the vabS gene have been identified. The characteristics determined by bioinformatics software and primer design were obtained. Primer set and probe specificity were verified by testing in software databases. The 100% sequence of the obtained primer set was found to overlap only with *L. anguillarum* in all bacteria. The probe was tested in single-chain blast, confirming its specificity. PCR and electrophoresis steps were performed on the genome with the obtained primer set and probe.
Keywords: Fish diseases, *Listonella anguillarum*, Primer design, Bioinformatics, Probe design

1. Introduction
Fish farming is an important sector that provides great profits because it is a healthy food source, employment creation, and an important export product. Production of different marine and freshwater fish species is growing each year. Because, fish are grown in enclosed areas such as pools and cages and stock densities are increasing steadily to improve productivity in the unit. This leads to the formation of unsuitable aquaculture conditions for fish and, consequently, to increased sensitivity to infections (Sakai, 1999). We think that it is extremely important to take prophylactic measures periodically in intensive aquaculture, especially against bacterial and viral diseases, and to apply the treatment methods consciously when necessary.

Some researchers have used the 16S rRNA gene region for the diagnosis of bacterial fish disease agents (Osorio et al., 1999), and some researchers have used polymorphism based studies such as PCR and RFLP to identify them (Zappulli et al., 2005). In addition, many researchers have been working on bacterial fish disease agents with PCR studies conducted with different methods. These studies have been reported to perform nested PCR (Gonzalez et al., 2003), multiplex PCR (Amagliani et al., 2009) and RAPD-PCR (Dalla Valle et al., 2002) methods. In addition to molecular-based studies, especially in the identification and identification of bacterial fish diseases, studies have also been carried out that indicate that specific primers are designed to work (Zlotkin et al., 1998; Önalan and Arabaci, 2016). Several studies have been carried out by designing primers and probes (Balta et al., 2010; Cheng, 2002; Vendrell, 2006; Nilsson and Strom, 2002; Toyama et al., 1994; Izumi and Wakabayashi, 2000; Urdaci et al., 1998).

In this study, it is aimed to perform primer design in molecular based studies to be performed for bacterial fish disease agents and to use Bioinformatics applications for this purpose. *L. anguillarum* Gram is a negative bacillus and has been isolated from almost all species of marine fish including sea bream and sea bass and trout cultured in fresh water (Austin and Austin, 2012). It often causes hemorrhages in various tissues and organs

including skin, skeletal muscles, liver, spleen and kidney (Roberts, 2012). In a study on Listonellosis, it was reported that the presence of asymptomatic fish on the farms was determined by the PCR method and the environmental condition appeared to be optimal for the bacteria (Cheng, 2002). In another study, it was reported that among the fish sharing the same environment, the injured fish were exposed to each other by fecal-oral route (Pinto et al., 2017). As a result of studies carried out by some researchers, it has been reported that the agent is also isolated from pool water, mud, sediment and aquatic equipment's besides fish in farms (Kusuda et al., 1991; Kitao et al., 1979). The best way to protect yourself from the disease is to avoid infection by working with eggs and rootstocks that do not carry the disease agent. For this purpose, molecular identification has large preliminaries (Vendrell, 2006).

2. Materials and Methods

Information on the *Listonella (Vibrio) anguillarum* agent to be used in the study was obtained from the National Center for Biotechnology Information (NCBI) website. Sequence data of the *L. anguillarum* agent were obtained with the Genbank number (Figure 1).

The genomic sequence data to be explored were blasted and the regions with the least similarity in all organisms were identified. The choice of genes over these regions is the first step to be taken so that the similarity rate in the next steps is lower, so that the primer and probes to be designed are more specific (Figure 2). As seen in Fig. 2, only 100% of the entire genome overlaps with *Listonella (Vibrio) anguillarum* with accession number LK021130.1 Genbank. However, it should be noted here that in the results on the right side, the gene is scanned from the regions where the ID factor is "0". Following the acquisition of these data, the data are transferred to the bioinformatics software and processed.

Screening of the *Listonella (Vibrio) anguillarum*-affecting genome with accession number LK021130.1 and individual assays of the genes revealed that the vabS gene had no other paired gene length suitable for primer and probe design. It is aimed to see the overlap rate with other bacteria's by performing selected genetic blast. For this, the gene sequence CLC bio (Qiagen) software was blasted and only *Listonella (Vibrio) anguillarum* appears to overlap the sequence 100%. As can be seen from the blast result, the sequence from the 600th to the last appears only to *Listonella (Vibrio) anguillarum*, while the sequence from the six hundredth order appears to be similar to the partially different bacterial species. Therefore, when designing the primer and expression probe, the first 600 bases later will enhance the primer's specificity, which we do not prefer.

Gene specific primers to be used in the study were determined using Primer3 ((http://bioinfo.ut.ee/ Primer3-0.4.0/primer3) and manual optimizations were performed. The characteristics of the obtained primer set are given in the table below (Table 1). Among the designed primers, the product size is 204 bp long and a banding at this length is considered as a good value for probe design. The designed primers were checked to see if they match the different bacterial species as a result of the blast. For this purpose, the specificity of the primers was determined using CLC Bio nBlast application (Fig. 3). As shown in Figure 3, the designed forward primer overlaps with *Listonella (Vibrio) anguillarum*, whereas the reverse primer sequence overlaps with 20 other complete polynucleotides, only *Listonella (Vibrio) anguillarum*, and the last 18 polymorphisms overlap with another bacterial species. Therefore, it does not appear to be a PCR-related problem for this bacterium species due to the non-overlapping of the entire primer. The probe, which will be created between the two designed primer regions, will also increase specify and possibly result in false positive results.

As raise PCR specified, we can be design a probe between two primers. For this reason, we use beacon designer. After select our PCR product region, in beacon can give us a probe as our determined features. And we can use that probe in PCR as a more specific oligonucleotide sequence. For probe design we need some features for example melting temperature must be rice 10 degree from primer melting temperature and PCR amplicon length must be 200 bp optimum length. A probe providing this conditions can be used for PCR. In this study we research a probe between two primers by Beacon. The result of probe sequence and properties is following (Table 2). The probe sequence obtained from beacon Bioinformatic software is blasted by beacon online. We see that all results of sequence blasting are *Listonella (Vibrio) anguillarum* (Fig. 4).

PCR amplification and amplicon were obtained from the PCR simulator using forward and reverse primers (Fig. 5). For this purpose, SnapGene 4.0.7 program was used and PCR was performed on primers and template DNA. By using bioinformatics software's you can see MW (125,231 Da) and your selected regions molecules number ($1ng=4.81x10^9$). After Stimulate PCR process, we need see our bands on electrophoresis (Fig. 6). Because of this reason, we are using stimulate electrophoresis by SnapGene software. We can see how we must be use agar percent in electrophoresis. And we can chance our optimizations by bioinformatics software's.

3. Results

The aquaculture sector has great importance in terms of economy and employment in our country which is covered with sea on three sides. The incidence of diseases in fishes grown in parallel with the development of aquaculture in our country is also increasing. Early isolation and identification of the causative agent is important

in terms of the treatment process.

In studies related to fish diseases, the use of bioinformatics software has great prominence as well as the great importance of routine biochemical and phenotypic studies in studies carried out in today's technology. This is because the testing of preliminary data and information of a planned worker and the possible risks to be taken into consideration provide a great advantage in terms of the sustainability of the work. If a PCR-based study is targeted, it is not possible to have the available primer or probe for each run. In such a case different specific primers or probes may need to be designed for each different run. In addition, early isolation and identification of the causative agent is important in terms of the treatment process. In these studies, which are mostly performed on PCR basis, disease agents are isolated and identified from 16S rRNA or specifically identified gene regions in bacteria. However, since the 16S rRNA gene locus does not differ greatly in terms of base length and sequence in the vast majority of bacteria, the use of bacterial specific genes is likely to give more specific results in terms of primer and probe. Bioinformatics applications are crucial in increasing the specificity of the study design and of minimizing the mistakes that can be faced in order to construct a molecular-based work in the field of aquatic diseases.

In this study, applications were made on *Listonella (Vibrio) anguillarum* case which is affecting Listonellozis disease which is also seen in Turkey. Using bioinformatics software, genomes with accession number LK021130.1 were scanned for genes with low homology. The vabS gene located on the genome has been found to have the most suitable sequence for this purpose. Next, the specific areas of the primers were determined on this gene, and the properties determined by bioinformatics software and primer design were obtained by loading the software. The characteristics determined between the two primer regions in the PCR amplicon region were designed using loaded software. The obtained forward-reverse primers and probe specificity were verified by testing in bioinformatics software databases. The primers obtained were found to overlap only 100% of all bacteria with *L. anguillarum* and no other similarity. The designed and optimized probe was characterized by a base of 23 bases in length and only tested in databases designed specifically for *L. anguillarum*. PCR and electrophoresis steps were also carried out on the genome with the obtained primer set and probe, and the study design including the agarose density ratio was concluded.

As intended in this study, some investigators have also shown that they have designed specific primer sets for their studies (Mata et al., 2004; Özkök et al., 2008). However, many researchers have also reported that they use primers specific to the 16S rRNA gene for the identification of bacteria (Önalan and Arabacı, 2016). It was also found that researchers who worked the same influence continued to work with the primer sets in the literature in the previous studies (Korun, 2016; Akayli and Durna, 2017). Some researchers have reported that PCR-based studies are more sensitive, especially for viral agents (Taksdal et al., 2001; Ortega et al., 2007; Albayrak and Ozan, 2010; Dopoza and Barja, 2002). As a result of this study, each researcher will determine his / her specific subject and design a specific primer or probe for his/her work, which will lead to more specific studies.

References

Akaylı, T., ve Durna, M., (2017). Kültür Levrek (*D. Labrax*) balıklarından izole edilen *Vibrio anguillarum* izolatlarının karakterizasyonu. *Kocatepe Vet. J.* (2017) 10(3):134-141.

Albayrak H, Özan E., (2010). Gökkuşağı alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) infeksiyöz pankreatik nekrozis ve infeksiyöz hematopoietik nekrozis virus enfeksiyonlarının varlığının araştırılması. *Ankara Üniv Vet Fak Derg*, 57, 125-129, 2010.

Amagliani, G., Omiccioli, E., Andreoni, F., Boiani, R., Bianconi, I., Zaccone, R., ... & Magnani, M., (2009). Development of a multiplex PCR assay for *Photobacterium damselae subsp. piscicida* identification in fish samples. *Journal of fish diseases*, 32(8), 645-653.

Austin, B., Austin, D. A., Austin, B., & Austin, D. A., (2012). Bacterial fish pathogens (p. 652). Heidelberg, Germany: *Springer*.

Balta, F., Sandalli, C., Kayis, S., & Ozgumus, O. B., (2010). Molecular analysis of antimicrobial resistance in *Yersinia ruckeri* strains isolated from rainbow trout (*Oncorhynchus mykiss*) grown in commercial fish farms in Turkey. *Bull. Eur. Ass. Fish Pathol*, 30(6), 211-219.

Cheng, C. P., Pogany, J., & Nagy, P. D., (2002). Mechanism of DI RNA formation in tombusviruses: dissecting the requirement for primer extension by the tombusvirus RNA-dependent RNA polymerase in vitro. *Virology*, 304(2), 460-473.

Dalla Valle, L., Ramina, A., Vianello, S., Belvedere, P., & Colombo, L., (2002). Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum). *The Journal of steroid biochemistry and molecular biology*, 82(1), 19-32.

Dopoza CP, Barja JL, (2002). Diagnosis and identification of IPNV in salmonids by molecular methods. In, Cunningham CO (Ed): Molecular Diagnosis of Salmonid Disease. pp. 23-48, *Kluwer Academic Publishers*, Notherlans.

Gonzalez SF, Osorio CR, Santos Y., (2003). Development of a PCR based Method for The Detection of *Listonella anguillarum* in Fish Tissues and Blood Samples. *Dis. Aquat. Org.* 2003; 55: 109-115.

Gürçay, M., Turan, T., & Parmaksız, A., (2013). Türkiye'de Kültürü Yapılan Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss* Walbaum, 1792) İnfeksiyöz Pankreatik Nekrozis Virus Varlığının Tespiti Üzerine Bir Araştırma. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 19(1).

Izumi, S., & Wakabayashi, H., (2000). Sequencing of gyrB and their application in the identification of *Flavobacterium psychrophilum* by PCR. *Fish Pathology*, 35(2), 93-94.

Kitao, T., Aoki, T., Iwata, K., (1979). Epidemiological study on streptococcicosis of cultured yellowtail (*Seriola quinqueradiata*) Distribution of Streptococcus sp. in sea water and muds around yellowtail farms, *Bulletin of the Japanese Society of Scientific Fisheries* 45, 567-572.

Korun, J., (2016). Kültür Levrek (*Dicentrarchus labrax*, L.) Balıklarından İzole Edilen *Vibrio harveyi* Suşlarının Antimikrobiyal Duyarlılıkları Üzerine Bir Çalışma. *Nevşehir Bilim ve Teknoloji Dergisi*, 411-421.

Kusuda, R., Kava, K., Salati, F., Banner, R., (1991). *Enterococcus seriolicida sp. nov.* a Fish Pathogen, *International journal of systematic Bacteriology* Vol. 41, No. 3 pp. 406-409.

Mata, A. I., Gibello, A., Casamayor, A., Blanco, M. M., Domínguez, L., & Fernández-Garayzábal, J. F., (2004). Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Applied and Environmental Microbiology*, 70(5), 3183-3187.

Nilsson, W. B., & Strom, M. S., (2002). Detection and identification of bacterial pathogens of fish in kidney tissue using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. *Diseases of aquatic organisms*, 48(3), 175-185.

Osorio, C. R., Collins, M. D., Toranzo, A. E., Barja, J. L., & Romalde, J. L., (1999). 16S rRNA Gene Sequence Analysis of *Photobacterium damselae* and Nested PCR Method for Rapid Detection of the Causative Agent of Fish Pasteurellosis. *Applied and Environmental Microbiology*, 65(7), 2942-2946.

Ortega C, Vega F Enríquez R., (2007). Occurrence of the ınfectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) farms in Hidalgo State, Mexico. *Bull Eur Ass Fish Pathol*, 27(3): 100-107 2007

Önalan, Ş., & Arabacı, M., (2016). Van, Bitlis, Muş ve Hakkari illerinde bulunan gökkuşağı alabalığı çiftliklerinden elde edilen *Lactococcus garvieae* izolatlarının fenotipik, serotipik ve genotipik farklılıklarının belirlenmesi. *Yüzüncü Yıl Üniversitesi. Fen Bilimleri Enstitüsü,* Doktora Tezi.

Özkök, S., Şen, S., & Ün, H., (2008). Kültürü yapılan Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss*) Bakteriyel Böbrek Hastalığı (BKD)'nın teşhisi. *Etlik Veteriner Mikrobiyoloji Dergisi*, 1.

Pinto, M. F., Baptista, T., & Afonso, C. C. N., (2017). Development of a new multiplex-PCR tool for the simultaneous detection of the fish pathogens *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi* and *Edwardsiella tarda*. *Aquatic Living Resources*, 30, 4.

Roberts R.J., (2012). *Fish Pathology, 4th edn*. Wiley-Blackwell, Chicester.

Sakai, M., (1999). Current research status of fish immunostimulants. *Aquaculture*, 172(1-2), 63-92.

Taksdal T, Dannevig BH, Rimstad E., (2001). Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT- PCR and cell culture isolation. *Bull Eur Ass Fish Pathol*, 21, 214-219.

Toyama, T., Kita-Tsukamoto, K., & Wakabayashi, H., (1994). Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. *Fish Pathology*, 29(4), 271-275.

Urdaci, M. C., Chakroun, C., Faure, D., & Bernardet, J. F., (1998). Development of a polymerase chain reaction assay for identification and detection of the fish pathogen *Flavobacterium psychrophilum*. *Research in Microbiology,* 149(7), 519-530.

Vendrell, D., Balcázar, J. L., Ruiz-Zarzuela, I., De Blas, I., Gironés, O., & Múzquiz, J. L., (2006). *Lactococcus garvieae* in fish: a review. *Comparative immunology, microbiology and infectious diseases*, 29(4), 177-198.

Zappulli, V., Patarnello, T., Patarnello, P., Frassineti, F., Franch, R., Manirin, A., Castagnaro, M. and Bargelloni, L., (2005). Direct identification of *Photobacterium damselae subsp. piscicida* by PCR-RFLP analysis. *Diseases of Aquatic Organisms* 65, 53-61.

Zlotkin, A., Eldar, A., Ghittino, C., & Bercovier, H., (1998). Identification of *Lactococcus garvieae* by PCR. *Journal of Clinical Microbiology*, 36(4), 983-985.

Table 1. Primer sequences and properties of Listonella (Vibrio) anguillarum vabS gene designed specifically.

Primer Name	Sequence	Len	Tm	GC	3' Compl
vabS_Forward	5'-GCAACATCATACGCCAGAGA-3'	20	59.83	50	0,00
vabS_Reverse	5'-GGAATGGCCGAATACATCAC-3'	20	60.16	50	0,00

Table 2. Designed probe in this study and its properties

Probe Name	Sequence (5'-3')	Tm	GC (%)	Self Compl
vabS_Probe	AACAGCACTACACAACCAACCAC	68,7	47,8	0

GenBank ▾

Vibrio anguillarum chromosome 1, strain NB10, complete sequence

GenBank: LK021130.1

FASTA   Graphics

Go to: ▽

```
LOCUS       LK021130            3119695 bp    DNA     circular BCT 04-FEB-2016
DEFINITION  Vibrio anguillarum chromosome 1, strain NB10, complete sequence.
ACCESSION   LK021130
VERSION     LK021130.1
DBLINK      BioProject: PRJEB5701
            BioSample: SAMEA3865288
KEYWORDS    complete genome; complete replicon.
SOURCE      Vibrio anguillarum (Listonella anguillarum)
  ORGANISM  Vibrio anguillarum
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
```

Figure 1. Acquisition of *Listonella (Vibrio) anguillarum* genomic data in NCBI.

Figure 2. Blast results of *Listonella (Vibrio) anguillarum* genomic data in NCBI

Figure 3. Blast results of forward and reverse primers (Left figure is forward primer, left figure is reverse primer).

Figure 4. Blast results of designed probe belong to *Listonella (Vibrio) anguillarum*.

Figure 5. Screening of PCR amplicon region with two primers designed in the study (SnapGene 4.0.7, original)
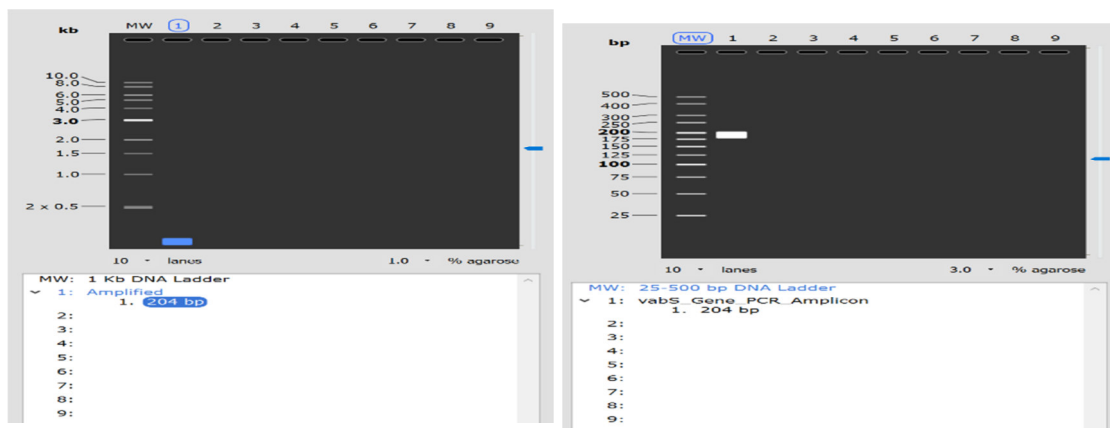

Figure 6. Electrophoresis screen of vabS gene PCR amplicon results with wrong and correct results.